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11th EDITION

Ananthanarayan and Paniker's Textbook of Microbiology

As per CBME Curriculum

Universities Press

Editor

Reba Kanungo

Ananthanarayan and Paniker's Textbook of Microbiology

11th EDITION

R Ananthanarayan (1913–1998)

Dr R Ananthanarayan graduated from Madras Medical College in 1940, and began his illustrious medical career in the Indian Army, serving as Quarter Master and Medical Officer in charge of wards in Sudan and Egypt. He also served in Iraq, Iran, Palestine, Syria, Malaya and Java. He was appointed Assistant Surgeon at the Government hospital in Calicut in 1946, moving to Stanley Medical College the following year. He obtained a Diploma in Bacteriology (DB) from London in 1951 and his PhD in 1953. For the next eight years, he worked as Professor of Bacteriology in the newly established Trivandrum Medical College, after which he assumed the post of Principal and Professor at Calicut Medical College. In May 1967, he was transferred to Kottayam Medical College from where he retired in April 1969. Retirement did not slow him down, however, and he served as Professor of Microbiology at Gulbarga Medical College till 1972. From 1972–1975, he served as Professor Emeritus at Trivandrum Medical College and Adviser to the Kerala Government on Laboratory Services.

During his career, Dr Ananthanarayan served as Examiner for Microbiology in different universities, was a member of several commissions and was awarded the Arun Banerjee Memorial Award in 1979. A Founding Member of the Indian Association of Pathologists and Microbiologists, he was elected President in 1976 and also served as President of the Association of Microbiologists in India in 1980. A greatly respected and revered teacher, he published more than 64 papers in reputed journals.

CK Jayaram Paniker (1932–2014)

Dr CK Jayaram Paniker graduated from Madras Medical College in 1955 and completed his postgraduate degree from All India Institute of Medical Sciences in 1962. During the initial phases of his career, he worked at the Pasteur Institute of India, Coonoor, Trivandrum Medical College and as Founder–Professor of Department of Microbiology at

Kottayam Medical College. He was Principal, Director and Head of the Department of Microbiology at Calicut Medical College and Dean, Faculty of Medicine, Calicut University.

Well known for his command over the English language and for his sparkling sense of humour, Dr Paniker performed yeoman service for Microbiology in India. He was the recipient of the BC Roy Award, and the Kerala Government honoured him with the post of Professor Emeritus in Microbiology. He headed many advisory committees and published several research papers, especially in the fields of typhoid and influenza.

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Reba Kanungo

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and
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ANANTHANARAYAN AND PANIKER'S TEXTBOOK OF MICROBIOLOGY, ELEVENTH EDITION

UNIVERSITIES PRESS (INDIA) PRIVATE LIMITED

Registered Office
3-6-747/1/A & 3-6-754/1, Himayatnagar, Hyderabad 500 029, Telangana, India
info@universitiespress.com, www.universitiespress.com

Distributed by
Orient Blackswan Private Limited

Registered Office
3-6-752, Himayatnagar, Hyderabad 500 029, Telangana, India

Other Offices
Bengaluru, Chennai, Guwahati, Hyderabad, Kolkata
Mumbai, New Delhi, Noida, Patna, Visakhapatnam

© Universities Press (India) Private Limited 2005, 2009, 2013, 2017, 2020

e-edition: First published 2021

eISBN 9789389211757

Cover image source: © Shutterstock #165299387. Virus 3D Image.
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Published by
Universities Press (India) Private Limited
3-6-747/1/A & 3-6-754/1, Himayatnagar, Hyderabad 500 029, Telangana, India

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Preface to the Eleventh Edition

To keep abreast of the rapidly changing global and local infectious disease spectra, the knowledge and practice of microbiology need to advance. The Competency-Based Medical Education (CBME) curriculum is aimed at integrating microbiology into the system-based approach to human disease. The eleventh edition of *Ananthanarayan and Paniker's Textbook of Microbiology* has been revised to address this restructuring of the curriculum and to make it better suited for the shorter course duration and system-based integration.

Great care has been taken to include the most recent advances in microbiology and infectious diseases and to do away with obsolete concepts and material. Chapters have been pruned without compromising on essential elements which have been presented in a lucid style and flow for an easy and enjoyable reading experience. Several clinical and laboratory images have been updated and line diagrams included for better visual impact and comprehension. Recent advances in disease detection, molecular diagnosis, quality control, infection prevention and control, public health and epidemiology and preventive strategies including national programmes have been brought up to date. An entire chapter (chapter 60) has been devoted to the essence of the competency-based integrated approach to system-wise infectious diseases. This chapter delves into the entire gamut of organisms involved in infectious diseases commonly affecting various systems of the human body and will pave the way for the study of pathogenic microorganisms as individuals and as groups. The traditional approach to the learning and understanding of the microbe ↔ host ↔ environment interaction, pathogenesis, clinical presentations, diagnosis, treatment and prevention of infections has been retained.

I must put on record my heartfelt gratitude to Mrs Desdemona Rashitha, a tutor in the Department of Microbiology, Pondicherry Institute of Medical Sciences (PIMS), for her untiring help and her skilled preparation and photography of media, culture plates, smears and biochemical reactions which were so valuable to this edition. I would also like to thank Dr Sheela Kuruvila, Head of the Department of Dermatology and Venereal Diseases,

PIMS, for contributing numerous photographs of clinical infections of the skin and soft tissue. I am also grateful to my esteemed colleagues, Dr Angeline Neeta Radjou, Professor of Surgery, and Dr Mary Kurien, Professor of ENT, PIMS, for contributing photographs from their personal collections. I would like to thank the TB division, IRL, Cuttack, Dr Sherly Antony, Research Centre, Pushpagiri Medical College, and Dr Isabella Princess, Apollo Hospitals, Chennai, for sharing images of automated laboratory equipment. Dr Sheela Devi's help in coordinating the CBME alignment is gratefully acknowledged. I would also like to extend my gratitude to Dr Shampa Anupurba, Professor and Head of Microbiology, BHU, Varanasi, for her valuable inputs, and the teaching faculty of the Department of Microbiology, PIMS, who have contributed, over several sessions, to the discussion of CBME in preparation for the ensuing academic year.

In an effort to update and make this classic textbook of microbiology relevant to the present context of competency-based teaching and learning methods for teachers and students, I have had the invaluable support of the editorial team at Universities Press, especially Dr Sudha Ganesan who was a constant support at every stage of the preparation of this edition, providing significant insights drawn from her vast technical knowledge and expertise. Mr Madhu Reddy has been a constant pillar of strength from behind the scenes, providing guidance throughout. Mrs Parvath Radha and Malini Gopalakrishnan, at the technical editing and proofreading stages, have contributed substantially to the finer nuances of publishing this textbook. Finally, I thank the numerous students and teachers of microbiology who provided feedback which was invaluable in fine-tuning the book.

I hope that readers find the competency-based approach of the book helpful and hope that they find this edition of value in understanding and appreciating the galactic subject that is microbiology.

Reba Kanungo

Preface to the First Edition

Many of the health problems in developing countries like India are different from those of developed countries. Bacterial diseases still play a considerable role in our country. Topics such as cholera and enteric diseases are important to us though only of less or academic interest to the developed countries. The increasing importance of the newer knowledge in immunology to health and disease is not adequately stressed in most of the extant textbooks. Virus diseases which are responsible for nearly 60 per cent of human illness require wider coverage. The general approach to the teaching of microbiology in our country has also been rather static. All these factors called for a textbook of microbiology more suited to countries like India.

We therefore undertook this endeavour based on our experience of teaching undergraduates and postgraduates for over two decades. We omitted the discipline of parasitology from our book since we already have an excellent textbook on the subject published in India.

This book has taken us over three years to write and over a year in publication. Naturally, we would be out of date to a certain and inevitable extent. We do not claim any perfection. On the contrary, we have requested medical students and teachers all over the country to write to us about any shortcomings and give us suggestions as to how to improve the book. We shall spare no pains in seeing that their valuable suggestions are given effect to in our second edition.

R Ananthanarayan
CK Jayaram Paniker

Note to Teachers

On adapting the eleventh edition of *Ananthanarayan and Paniker's Textbook of Microbiology* to the Competency-Based Medical Education (CBME) curriculum

Microbiology is a vast subject that has traditionally been taught as general microbiology, immunology, bacteriology, virology, parasitology, mycology and applied microbiology (added on a few years ago).

With the introduction of competency-based teaching and learning, the traditional method of teaching microbiology will have to change. While general microbiology and immunology continue as separate competencies in this revised structure, the emphasis in the former is on sterilisation and disinfection as applied to health care settings, whereas in applied immunology, the emphasis is on immunological disorders (immunodeficiency, autoimmune diseases, etc.). In this approach, there is scope for integrating immunology with pathology. Added attention is to be paid to the application of these subjects in diagnostic tests.

The eleventh edition of *Ananthanarayan and Paniker's Textbook of Microbiology* elaborately and comprehensively deals with system-wise infections in a single chapter (chapter 60), addressing all the systems according to the CBME curriculum. It is suggested that this form the dashboard for subsequent chapters. The individual organisms (bacteria, viruses, fungi and parasites) pertaining to each system may be addressed in detail within that system. For example, in the competency on CVS and bloodstream infections, the common etiological agents of such infections can be listed. The major organisms of each group may then be addressed in detail in their individual chapters. There will, of course, be a lot of overlap as organisms affecting multiple systems will crop up time and again and will have to be discussed several times. To save on time and resources, it is suggested that the main causative agents be addressed under the particular system they affect and recapitulated in subsequent chapters. For example, *S. pneumoniae* may be taught in detail under infections of the respiratory system and then simply listed or reiterated while discussing meningitis. We have taken time to work on the template provided here as an example of organisms to be taught in each system (the teaching of organisms that

require detailed explanation is best left to the instructor's discretion).

- **CVS and bloodstream infections**

  Group A streptococci, other streptococci, enterococci and the HACEK group

  Others (viral coxsackie)

  Hemolytic anemia—HUS organisms, CMV, EBV, mumps, measles and arboviruses

  Parasitic infections—malaria, leishmaniasis, trypanosomiasis, *Diphyllobothrium* and hookworm infections

- **Gastrointestinal and hepatobiliary tract infections**

  Diarrhea—cholera, enteric fever, diarrheagenic *E. coli*, non-typhoidal salmonellosis and antibiotic-associated diarrhea

  Viral gastroenteritis—rotavirus, norovirus, calicivirus, astrovirus, etc.

  Parasitic agents—coccidian parasites and *Strongyloides*

  Dysentery—bacterial (*Shigella*, *Campylobacter*, *V. parahaemolyticus*) and amoebic dysentery (*E. histolytica)*

  Intestinal parasites—nematodes and trematodes

  Food poisoning—*Staphylococcus*, *Clostridia*, *Bacillus cereus* and mycotoxins

  Viral hepatitis—HAV, HEV, HBV, HDV, HCV, yellow fever and CMV

  Other causes of hepatitis—*Leptospira*

- **Musculoskeletal system—skin and soft tissue infections**

  Clostridial infections and staphylococcal infections, anthrax and leprosy

  Exanthematous viral diseases (measles, rubella, varicella–zoster, parvovirus, HHV-6, -7 and -8, mumps, papovaviruses, poxvirus)

  Tissue nematodes—*Onchocerca*, *Loa loa*, *Mansonella* and *Dracunculus*

  Superficial and subcutaneous fungal infections

  Actinomycetes and *Nocardia*

- **Central nervous system infections**

Neisseria meningitidis, Haemophilus influenzae, Streptococcus pneumoniae, TB meningitis*, Cryptococcus*

Enteroviruses, poliovirus, coxsackievirus, echovirus, mumps virus and other viruses, and spirochetes (*T. pallidum* and *Leptospira*)

Encephalitis—Herpes viruses (HSV-1 and -2), encephalitis group of arboviruses (JE and West Nile viruses)

Rabies, Nipah virus, slow viruses

Primary amoebic encephalitis—neurocysticercosis and toxoplasmosis

- **Respiratory infections**

URI—*C. diphtheriae, Streptococcus* spp.*, Staphylococcus* spp., influenza virus, adenovirus, coronavirus and rhinovirus

LRI—*S. pneumoniae*, *H. influenzae*

Atypical pneumonia—*Mycoplasma, Chlamydophila* (psittacosis and pneumonia) and *Legionella*

Tuberculosis—*Mycobacterium tuberculosis* and non-tuberculous mycobacteria

Bordetella pertussis

Influenza virus, parainfluenza and RSV

Aspergillus spp. and pneumocystis

- **Genitourinary system and sexually transmitted infections**

Gonorrhea and non-gonococcal urethritis (*Chlamydia*, *Mycoplasma*, *Ureaplasma* and *Trichomonas vaginalis* and bacterial vaginosis)

T. palladium, *H. ducreyi*, *Lymphogranuloma venereum* and granuloma inguinale

Genital herpes

Urinary tract infections—*E. coli*, *K. pneumoniae*, *Proteus* spp. and *Enterococcus* spp., *Staphylococcus saprophyticus* and *S. agalactiae*

- **Zoonotic diseases and arthropod- and rodent-borne infections**

Brucellosis, plague, rickettsial infections, scrub typhus and leptospirosis

Miscellaneous zoonoses—relapsing fever and bovine TB

Vector-borne diseases—dengue, chikungunya, KFD and Crimean Congo hemorrhagic fever

- **Miscellaneous infections (opportunistic infections)**

Mycobacterium tuberculosis, MOTT complex, *Legionella pneumophila*, cytomegalovirus

HHV-8 (KSHV), human polyomavirus-2 (JC virus), yeasts (*Candida*, etc.), moulds (*Aspergillus,* etc.), *Histoplasma capsulatum* and *Pneumocystis jirovecii*

- **Health care-associated infections**
- **Bacteriology of milk, water and air**
- **National health programmes—**IDSP, NTEP, etc.
- **AETCOM modules as applicable to microbiology**

To sum up, a textbook can only serve to help faculty teach a subject in a manner that will logically explain and structure the subject to be delivered to the student, as per the recommended guidelines. The aim is to convey the integrations and open up the subject to the actual applications in clinical settings. Faculty are therefore invited to navigate the TOC and content, which has been presented to the level of the undergraduate, to suit the method that best serves this purpose for the student.

General Learning Objectives

(As per the CBME curriculum)

The following are the section-wise objectives of this book for students of microbiology:

Sr. No.	Learning objective
Part I GENERAL MICROBIOLOGY	
1.	Describe the morphology, physiology, growth, nutrition and genetics of bacterial agents
2.	Describe the methods used for the detection of bacterial agents, including the application of molecular techniques
3.	Perform Gram staining and ZN staining for the detection of various bacterial agents
4.	Classify and describe the different agents of sterilisation and disinfection
5.	Discuss the application of different methods of sterilisation and disinfection in the laboratory and in clinical and surgical practice
Part II IMMUNOLOGY	
1.	Discuss the role of microbial agents in the causation of disease
2.	Explain the immune mechanisms in health
3.	Describe the host immune response in microbial infections
4.	Discuss the mechanisms of immunological disorders like hypersensitivity, autoimmune disorders and immunodeficiency states
5.	Outline the methods for the detection of immunological disorders
6.	Describe the immunological mechanisms of transplantation
7.	Discuss tumour immunity
Part III BACTERIOLOGY	
1.	Discuss the role of normal flora in health and disease

Sr. No.	Learning objective
2.	Discuss the mechanisms of drug resistance, methods of antimicrobial susceptibility testing and monitoring of antimicrobial therapy
3.	Describe the etio-pathogenesis, clinical features and diagnostic modalities of the bacterial causes of: Rheumatic fever and infective endocarditis Anemia Diarrhea, dysentery and food poisoning Enteric fever Acid peptic disease (APD) Anaerobic infections Bone and joint infections Skin and soft tissue infections Meningitis and encephalitis Upper and lower respiratory tract infections Genitourinary tract infections Sexually transmitted infections Zoonotic infections
4.	Identify the various bacterial agents causing infection
Part IV VIROLOGY	
1.	Describe the general properties and classification of viral agents
2.	Discuss the pathogenesis and host response in viral infections
3.	Describe the modalities for the laboratory diagnosis of viral infections
4.	Discuss in brief antiviral agents and immunoprophylaxis for viral infections
5.	Describe the etio-pathogenesis, clinical features and laboratory diagnosis of viral infections like: Diarrhea and dysentery Viral hepatitis Skin and soft tissue infections Meningitis and encephalitis

Sr. No.	Learning objective
	Upper and lower respiratory tract infections Genitourinary tract infections HIV and other sexually transmitted infections Zoonotic infections Opportunistic infections Viral associated malignancy
Part V MYCOLOGY	
1.	Discuss the general properties and classification of fungal agents
2.	Describe the etio-pathogenesis, clinical features and laboratory diagnosis of fungal infections like: Cutaneous and subcutaneous infections Systemic infections Opportunistic infections
3.	Outline the modalities for the laboratory diagnosis of fungal infections
4.	Briefly discuss the anti-fungal agents
Part VI CLINICAL MICROBIOLOGY AS APPLIED TO INFECTIOUS DISEASES	
1.	Enumerate the bacterial, viral and fungal agents of various infectious diseases
2.	Outline the laboratory diagnosis of important infectious diseases including the choice of test, interpretation and recent advances
3.	Discuss health care-associated infections
4.	Describe in brief infection control measures
5.	Demonstrate infection control measures including hand hygiene and use of personal protective equipment
6.	Describe the etiological agents of emerging and re-emerging infections
7.	Describe the bacteriology of water, air, milk and food

Sr. No.	Learning objective
8.	Briefly describe the role of National Health Programmes in the prevention of common infectious diseases
9.	Discuss briefly quality control in the microbiology laboratory

Part I:
General Microbiology

Part I: General Microbiology

Chapter 1

Introduction to Microbiology

Chapter navigation

- Koch's postulates
- Phylogenetic classification
- Adansonian classification
- Molecular or genetic classification

INTRODUCTION

Medical microbiology is the study of microorganisms (bacteria, viruses, fungi, protozoa and helminths) and infectious protein particles (prions) that infect humans, the diseases they cause, and the diagnosis, prevention and treatment of such diseases. It also deals with the response of the human host to microbes and their antigens. Microorganisms are found abundantly almost everywhere in the environment, from mountaintops to the ocean bed. They have an intimate relationship with humans in that some colonise the human body while others cause disease. A large proportion of microbes make up the normal human gut flora and exist symbiotically, providing their host with essential elements required for a healthy life. Microbiomes are increasingly being researched to study their effects on human health and disease. Microorganisms have a great impact on all forms of life on this planet. They also play a vital role in agriculture and in the brewing industry.

HISTORICAL PERSPECTIVE AND NOTABLE MILESTONES IN MICROBIOLOGY

As microbes are invisible to the unaided eye, definitive knowledge about them had to await the development of the microscope. The credit for having first observed and reported bacteria goes to Antonie van Leeuwenhoek of Delft, Holland—a draper whose hobby was grinding lenses and observing

diverse materials through them (Fig. 1.1a).

Even before the microbial cause of infections had been identified, **Ignaz Philipp Semmelweis** of Vienna had independently concluded in 1846 that puerperal sepsis was contagious. Semmelweis also established that the infection was transmitted by doctors and medical students attending on women in labour in the hospital. He introduced the concept of **antisepsis**, which he demonstrated by washing his hands in chlorinated lime solution. Sadly, his observations were rejected by the medical fraternity; he died in an asylum, ironically, due to an infection—the very thing he had sought to combat all his life.

Though immunisation using the powdered dry scab of cowpox lesions as snuff was in practice in countries like China (during the period of the Ming Dynasty), India and Africa before the 18th century, it was **Edward Jenner** (1749–1823), an English physician, who paved the way for vaccines. As a country doctor, Jenner had heard of milkmaids becoming immune to smallpox after contracting cowpox. Seeing a connection, Jenner developed the smallpox vaccine from fluid taken from a cowpox blister and laid the foundation for protective immunity.

Louis Pasteur (1822–95) contributed significantly to the development of microbiology as a scientific discipline (Fig. 1.1b). His important contributions to the field include:

- Techniques of sterilisation and development of the steam steriliser, hot air oven and autoclave
- Knowledge of the growth needs of different bacteria
- Knowledge of anthrax
- Attenuation of chicken cholera bacilli
- Vaccines for hydrophobia (rabies) and anthrax

Pasteur accidentally observed that a culture of chicken cholera bacillus that was left on the bench for several weeks lost its pathogenic property but retained its ability to protect birds against subsequent infection. This led to the discovery of the process of attenuation and the development of live vaccines. He attenuated cultures of the anthrax bacillus by incubating them at a high temperature (42–43°C) and proved that inoculation of such cultures in animals induced specific protection against anthrax. It was Pasteur who coined the term '**vaccine**' for these cultures.

An immediate application of Pasteur's work was the introduction of

antiseptic techniques in surgery by Joseph Lister (1867), which resulted in a pronounced drop in mortality and morbidity due to surgical sepsis. Lister's antiseptic surgery involving the use of carbolic acid was a milestone in the evolution of surgical practice from the era of 'laudable pus' to modern aseptic techniques.

While Pasteur laid the foundations of microbiology in France, Robert Koch (1843–1910) perfected bacteriological techniques in Germany (Fig. 1.1c).

His works include:

- Studies on the culture and life cycle of the anthrax bacillus
- Staining techniques
- Methods of obtaining bacteria in pure culture using solid media
- The discovery of the tubercle bacilli (1882)
- The discovery of *Vibrio cholerae* (1883)

At the time, causative agents of various infectious diseases were being reported by different investigators in such profusion that it became necessary to introduce criteria for proving that a microorganism isolated from a disease was indeed causally related to it. These criteria were enunciated by Robert Koch and are known as Koch's postulates. According to Koch, a microorganism can be accepted as the causative agent of an infectious disease only if the following conditions are satisfied:

Koch's postulates

- The bacterium should be constantly associated with the lesions of the disease.
- It should be possible to isolate the bacterium in pure culture from the lesions.
- Inoculation of such a pure culture into suitable laboratory animals should reproduce lesions of the disease.
- It should be possible to re-isolate the bacterium in pure culture from the lesions produced in experimental animals.
- An additional criterion introduced subsequently requires that specific antibodies to the bacterium should be demonstrable in the serum of patients suffering from the disease.

Though it may not always be possible to satisfy all the postulates in every case, they have proved extremely useful in sifting doubtful claims made regarding the causative agents of infectious diseases.

Roux and Yersin (1888) identified a new mechanism of pathogenesis when they discovered the diphtheria toxin. Similar toxins were identified in tetanus and some other bacteria. These toxins were found to be specifically neutralised by their antitoxins.

Paul Ehrlich, who studied toxins and antitoxins in quantitative terms, laid the foundation for biological standardisation (Fig. 1.1d)

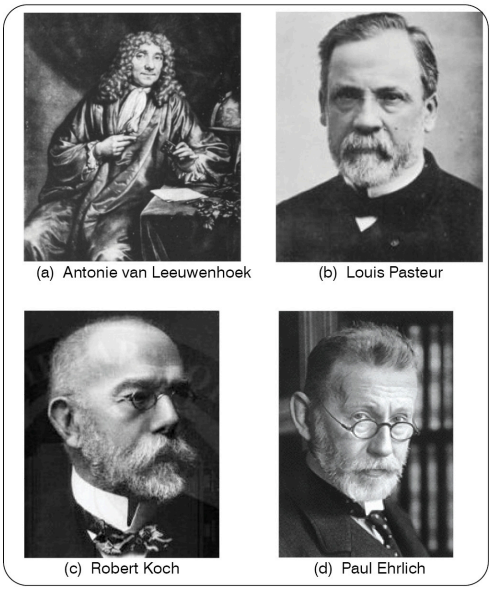


Fig. 1.1 Some eminent microbiologists whose contributions laid the foundations of microbiology, concepts of infectious disease and antisepsis

Ernst Ruska (1934) developed the electron microscope, enabling the visualisation of viruses. The development of tissue culture techniques permitted the cultivation of viruses.

Immunochemistry for the study of immunity had to await advances in protein chemistry. The pioneering work of Karl Landsteiner laid the foundation for immunochemistry. In 1955, Niels Jerne proposed the natural selection theory of antibody synthesis, which attempted to explain the chemical specificity and the biological basis of antibody synthesis, signifying a return to the original views of antibody formation proposed by Ehrlich (1898). Frank Burnet (1957) modified this into the clonal selection theory, a concept which, with minor alterations, holds true even now. The last few decades have witnessed an explosion of conceptual and technical advances in immunology. Immunological processes in health and disease are now better understood following the identification of the two components of immunity: a) humoral or antibody-mediated processes and

b) cellular or cell-mediated processes.

Antibiotic Era

An antibacterial substance was accidentally discovered by Alexander Fleming in 1928. He found that the fungus Penicillium produces a substance that destroys staphylococci. He published his findings in 1929, for which he was awarded the Nobel Prize. Florey, Chain and their team at Oxford isolated the active substance 'penicillin'. Its subsequent mass production during the Second World War saved the lives of many wounded soldiers. This was the beginning of the antibiotic era. Other similar antibiotics were discovered in rapid succession.

With the development of a wide variety of antibiotics against the whole spectrum of pathogenic bacteria and the advent of vaccines against some bacterial and viral diseases, expectations were raised about the elimination of all infectious diseases. The global eradication of smallpox inspired visions of similar campaigns against other major infections. But soon, the development of antibiotic resistance in bacteria presented serious difficulties.

In 1981, AIDS was identified in the USA before it went on to become a global pandemic. Since then, new and emerging infectious diseases have appeared from time to time—the Nipah virus in Kerala, Zika virus in South America, Mers-CoV in the Middle East and most recently, the coronavirus outbreak in the Wuhan district of China—proving that controlling microbes is far more difficult than was imagined.

Role of Medical Microbiology

Apart from obvious benefits such as specific methods of diagnosis, prevention and control of infectious diseases, medical microbiology has:

- contributed to scientific knowledge and human welfare in many other ways and has been employed as a model for the study of **genetics and biochemistry**.
- contributed to the unravelling of the genetic code and other mysteries of biology at the molecular level. Bacteria and their plasmids, yeasts and viruses are routinely employed as vectors in recombinant DNA technology.

- made available precious information and powerful techniques for **genetic manipulation and molecular engineering**. Used wisely and well, this information stands to benefit all life forms.

For the discoveries and innovations that became milestones in the field of microbiology, a number of scientists have been awarded the Nobel Prize in Medicine and Physiology. Some of these Nobel laureates and their contributions to the field of microbiology are listed in Table 1.1. Their work is testament to the positive contribution made to human health by the science of microbiology.

Other notable discoveries that paved the way for molecular microbiology are:
- The sequencing of DNA by W. Gilbert and F. Sanger
- The invention of the polymerase chain reaction by Kary Mullis in 1983
- The first whole genome sequence of a microbe (H. influenzae), published by The Institute of Genetic Research (TIGR) in 1995

Table 1.1 Some notable Nobel laureates and their contributions to the science of microbiology

Year	Nobel laureate	Work
1902	Ronald Ross	Malaria
1905	Robert Koch	Tuberculosis
1908	P. Ehrlich and E. Metchnikof	Immunity
1928	Charles Nicolle	Typhus
1930	Karl Landsteiner	Discovery of human blood groups
1945	A. Fleming, E. Boris Chain and Howard Walter Florey	Penicillin
1951	Max Theiler	Yellow fever
1952	Selman A. Waksman	Streptomycin
1954	Franklin Enders, T. H. Weller and F. C. Robbins	Poliomyelitis growth in tissue
1960	F. M. Burnet and P. B. Medawar	Acquired immunological tolerance
1966	Peyton Rous	Tumour-inducing viruses
1969	M. Delbrück, A. D. Hershey	Replication mechanism and the

	and S. E. Luria	genetic structure of viruses
1975	D. Baltimore, R. Dulbecco and H. Martin Temin	Interaction between tumour viruses and the genetic material of the cell
1976	Baruch S. Blumberg and D. Carleton Gajdusek	New mechanisms of infectious disease dissemination and Australia antigen
1980	Baruj Benacerraf, Jean Dausset and George D. Snell	Immunological regulation by cell surface
1984	Niels K. Jerne, Georges J. F. Köhler and César Milstein	Control of immune system and monoclonal antibodies
1987	Susumu Tonegawa	Generation of antibody diversity
1989	J. Michael Bishop and Harold E. Varmus	Origin of retroviral oncogenes
1996	Peter C. Doherty and Rolf M. Zinkernagel	Specificity in cell-mediated immune defence
1997	Stanley B. Prusiner	Prions
2005	Barry J. Marshall and J. Robin Warren	*Helicobacter pylori*
2008	Harald Hausen and Françoise Barré-S. and L. Montagnier	Human papilloma viruses and human immuno-deficiency virus
2011	Bruce A. Beutler, Jules A.Hoffmann and Ralph M. Steinman	Activation of innate immunity, and the dendritic cell and its role in adaptive immunity
2015	William C. Campbell and Satoshi Ömura	Discovery of a novel therapy against roundworm infection
2015	Youyou Tu	Discovery of a novel therapy against malaria
2018	James P. Allison and Tasuko Honjo	Though not directly related to microbiology, their work explained the process of negative inhibition of immunity in cancer therapy

CLASSIFICATION, NOMENCLATURE AND TAXONOMY

The primary purpose of classifying organisms is to enable easy

identification. Classification systems aim to group organisms with similar properties and to separate those that are different. The basic taxonomic unit in bacteria is the species. Species differ from one another in several features, which are determined by genes.

Bergey's Manual of Determinative Bacteriology is widely accepted for its systematic classification of microorganisms. The information in the manual is updated frequently to include new organisms or changes in nomenclature.

Bacterial taxonomy comprises three components:

1. Classification, or the orderly arrangement of units; a group of units is called a taxon (plural: **taxa**) irrespective of its hierarchic level
2. Identification of an unknown organism with a defined and named unit
3. Nomenclature or the naming of units

Bacterial Classification and Nomenclature

Carolus Linnaeus introduced the hierarchical system of classification—domain, kingdom, phylum, class, order, family, genus and species.

A kingdom is divided successively as follows:

Kingdom → Division → Class → Order → Family → Tribe → Genus → Species

The scientific name of an organism usually consists of two words, the first being the name of the genus and the second that of the species (e.g., Bacillus subtilis). This method of nomenclature was introduced by Carolus Linnaeus. The generic name is usually a Latin noun. The specific epithet is an adjective or noun and indicates some property of the species (e.g., albus, meaning white), the animal in which it is found (e.g., suis, meaning pig), the disease it causes (e.g., tetani, for tetanus), the person who discovered it (e.g., welchii, after Welch) or the place of its isolation (london). The generic name always begins with a capital letter and the species (specific epithet), with a small letter, even if it is the name of a person or place (e.g., Salmonella london).

Further classification into subspecies and serovariants include different serotypes within the species based on the organism's antigenic properties, e.g., generic name Salmonella; specific name enterica; subspecies enterica; serotype Typhi (i.e., Salmonella enterica enterica Typhi).

The properties of a population of bacteria are studied in toto and not with respect to an individual bacterium or microorganism, as is done for other life forms.

- A **clone** of a bacteria is the population derived by binary fission from a single cell.
- A single bacterial **colony** represents a clone. Though all the cells in a clone are expected to be identical in all respects, a few of them may show differences due to mutation.
- **Strain** is the population of bacteria derived from a particular source, such as from a patient.

Though the general absence of sexual reproduction in bacteria serves to keep their characteristics constant, bacteria possess several features that contribute to some degree of heterogeneity in their populations. Their short generation time and high rate of mutation lead to the presence, in any population, of cells with altered characteristics. Methods of genetic exchange such as transformation, transduction and conjugation cause differences in character.

Microorganisms are classified based on several characteristics which help in their identification. The different approaches to classification include phylogenetic classification, Adansonian classification, molecular or genetic classification and intraspecies classification.

1. ***Phylogenetic classification*** The hierarchical classification represents a branching, tree-like arrangement, one characteristic being employed for division at each branch or level. This system is called phylogenetic because it **implies an evolutionary arrangement** of species. In this system, some characteristics are arbitrarily given special weightage. Depending on the chosen characteristic, the classification would yield different patterns. While classification based on a weighted characteristic is a convenient method, it has the serious drawback that these characteristics may not be stable. Fermentation of lactose, for example, is not an essential and permanent characteristic. It may be acquired or lost, upsetting the system of arrangement. Based on the ribosomal RNA, 16S rRNA and 23S

rRNA gene sequences, phylogenetic relatedness among different bacteria can be revealed.

2. ***Adansonian classification*** It takes into account all the **characteristics expressed at the time of study** (Fig. 1.2). The availability of computers has widened the scope of this system by permitting the comparison of very large numbers of properties of several organisms at the same time. This is known as **numerical taxonomy**.
3. ***Molecular or genetic classification*** This is based on the **degree of genetic relatedness of different organisms**. Since all the properties of an organism are ultimately based on its genes, this classification is said to be the most natural or fundamental method. **DNA relatedness** can be tested by studying the nucleotide sequences of DNA and by DNA hybridisation or recombination methods. The nucleotide base composition and base ratio, i.e., adenine–thymine (A–T):guanine–cytosine ratio (G–C), remains constant for members of the same species but varies widely among different groups of microorganisms. Molecular classification has been employed more with viruses than with bacteria.
4. ***Intraspecies classification*** For diagnostic or epidemiological purposes, it is often necessary to subclassify bacterial species. This may be based on:

    - biochemical properties (**biotypes**),
    - antigenic features (**serotypes**),
    - bacteriophage susceptibility (**phage types**) and
    - production of bacteriocins (**colicin types**).

A species may be divided first into groups and then into types, for example, group A streptococci are subclassified into M,T and R types.

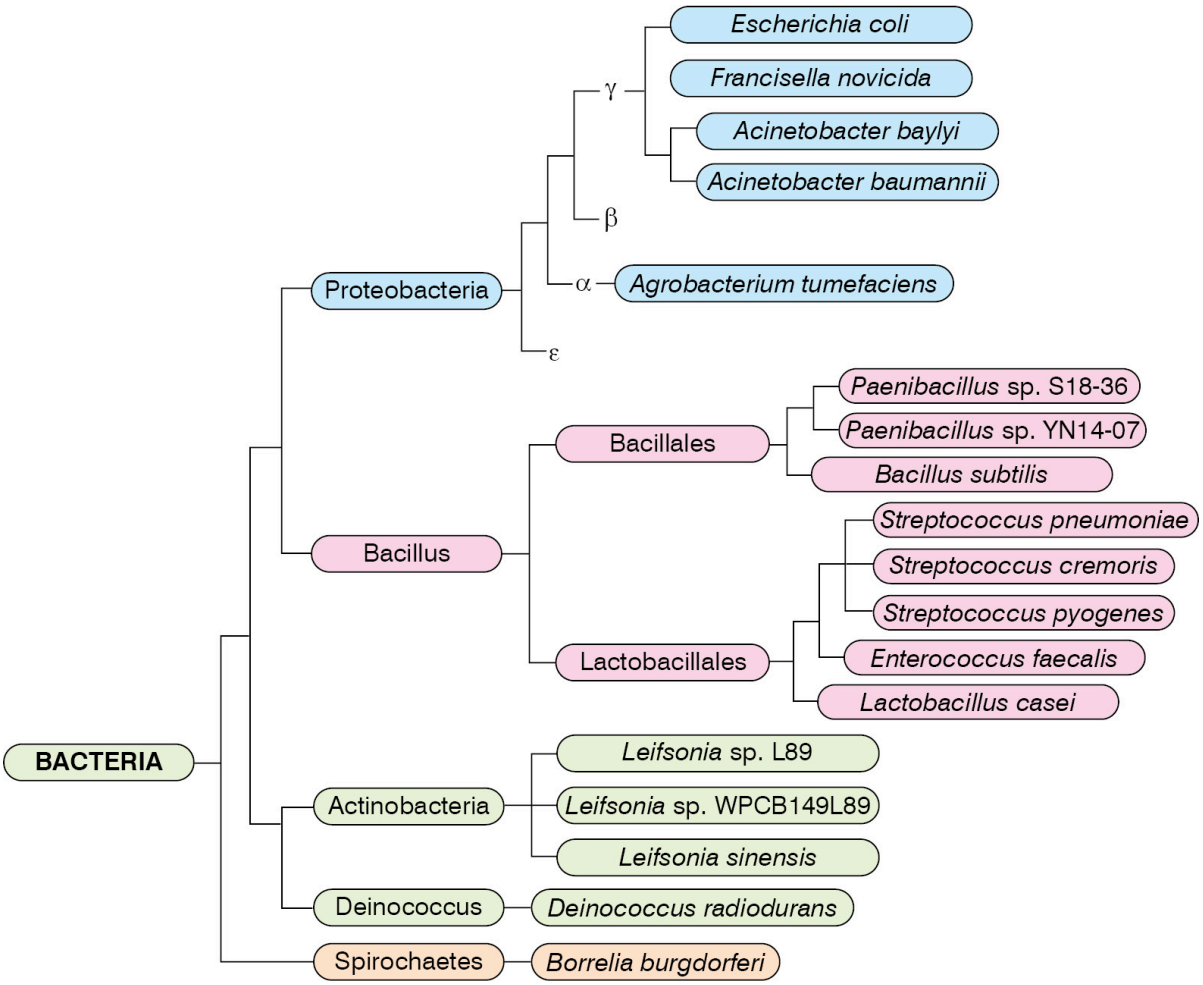


Fig. 1.2 Adansonian classification (*Source*: Bryksin AV, Matsumura I. Rational design of a plasmid origin that replicates efficiently in both gram-positive and gram-negative bacteria. PLoS ONE, 2010; 5(10): e13244. https://doi.org/10.1371/journal.pone.0013244)

Molecular epidemiology

Greater discrimination in intraspecies typing has been achieved by the application of newer techniques from immunology, biochemistry and genetics. Investigations of epidemiology and pathogenesis using these techniques are collectively referred to as **molecular epidemiology**. The methods used are:

1. **Phenotypic** (study of expressed characteristics) methods, which include electrophoretic typing of bacterial proteins and immunoblotting.
2. **Genotypic** (direct analysis of genes, chromosomal and extrachromosomal DNA) methods, which include plasmid profile analysis, restriction endonuclease analysis of chromosomal DNA with

Southern blotting, polymerase chain reaction and nucleotide sequence analysis.

Type cultures

As a point of reference, type cultures of bacteria are maintained in international reference laboratories. These type cultures contain representatives of all established species and the variants of these organisms that have been characterised. The original cultures of any new species described are deposited in type collections. They are made available by the reference laboratories to other workers for study and comparison. The important type collection centres in the world currently are:

- **ATCC (American Type Culture Collection)**
- **NCTC (National Collection of Type Cultures, Public Health England)**

Key points

- Over centuries, the experiments and work of a number of individuals from many different countries have provided a scientific basis to the study of diseases.
  i. Louis Pasteur (1822–1895) discovered methods of sterilisation and developed methods for the culture of microbes. He showed that microorganisms cause disease and established the principles of immunisation.
  ii. Joseph Lister (1827–1912) introduced 'antisepsis', wherein he sprayed the patient and operating field with carbolic acid.
  iii. Robert Koch (1843–1910) defined the criteria used to attribute a disease to a bacterium (Koch's postulates):
    - The bacterium must be found in all cases of the disease; their distribution in the body should correspond to that of lesions observed.
    - The bacterium should be isolated from the host and grown in a pure culture for several generations.
    - The bacterium should reproduce the disease in other susceptible animals.
    - The bacterium should be isolated in pure culture from lesions in infected animals.

    (An additional postulate, not formulated by Koch, was added later—a specific antibody to the organism should develop during the course of the disease.)
  iv) Ruska (1934) developed the electron microscope, enabling the visualisation of the microbes we now call viruses. The development of tissue culture techniques has permitted the cultivation of viruses.

v) Paul Ehrlich (1854–1915) was a pioneer in the study of antitoxin and toxin neutralisation.

vi) Sir Alexander Fleming (1881–1955) discovered that the fungus Penicillium produces a substance, penicillin, that destroys staphylococci; this discovery led to the formulation of other antimicrobials.

- Taxonomy is the science of classification. Classification of microorganisms is based on their arrangements into related groups or taxa.
- Since all organisms are classified primarily to enable easy identification, all classification systems aim to group organisms with similar properties and to separate those that are different. However, the best classification schemes are those that are based on evolutionary relatedness.
- The basic taxonomic unit of bacteria is species; two species differ from one another in several features determined by genes.

Questions

Short Answers

1. Robert Koch
2. Louis Pasteur
3. Joseph Lister
4. Koch’s postulates
5. Basis of bacterial classification

Part I: General Microbiology

Chapter 2

Morphology and Physiology of Bacteria

Chapter navigation

- Optic methods to visualise microorganisms
- Bacterial cell structure
- Staining techniques
- Growth and multiplication of bacteria
- Bacterial nutrition
- Bacteriocin

INTRODUCTION

Microorganisms are a diverse group of unicellular organisms that can be categorised into several distinct classes. Belonging to neither the plant nor the animal kingdom, these organisms are classified under a third kingdom, Protista. The kingdom Protista is further divided into:

- **Prokaryotes,** which are unicellular without true branching (except the actinomycetales).

Table 2.1 Some differences between prokaryotic and eukaryotic cells

Characteristic	Prokaryotes	Eukaryotes
Nucleus	Not well-defined	Well-defined
Nuclear membrane	Absent	Present
Nucleolus	Absent	Present
Deoxyribonucleoprotein	Absent	Present
Chromosome	One (circular) DNA molecule	More than one DNA molecule
Mitotic division	Absent	Present
Cytoplasm		

Cytoplasmic streaming	Absent	Present
Pinocytosis	Absent	Present
Mitochondria	Absent	Present
Lysosomes	Absent	Present
Golgi apparatus	Absent	Present
Endoplasmic reticulum	Absent	Present
Chemical composition		
Sterols	Absent	Present
Muramic acid	Present	Absent

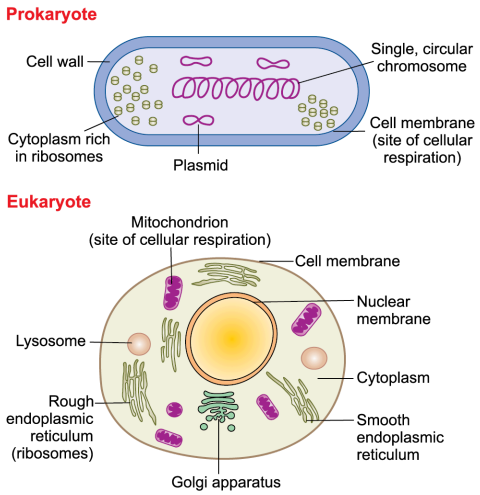


Fig. 2.1 Prokaryotic and eukaryotic cells

- **Eukaryotes,** which are multicellular and distinct from prokaryotes (Table 2.1).

Bacteria and blue-green algae are prokaryotes, while fungi, other algae, slime moulds and protozoa are eukaryotes (Fig. 2.1).

The size of microorganisms is expressed in microns (or micrometre, i.e., μm), the unit of measurement in bateriology.

The limit of resolution of the unaided eye is about 200 microns. Bacteria, being much smaller, can be visualised only under magnification; hence they can be seen only under a microscope. **Bacteria of medical importance generally measure 0.2–1.5 μm in diameter and about 3–5 μm in length.**

1 micron (μ) or micrometre (μm) = one-thousandth of a millimetre

1 millimicron (mμ) or nanometre (nm) = one-thousandth of a micron or one-millionth of a millimetre
1 Angstrom unit (Å) = one-tenth of a nanometre

OPTIC METHODS TO VISUALISE MICROORGANISMS

MICROSCOPY

The morphological study of bacteria requires the use of microscopes. **Antonie van Leeuwenhoek** was the first to observe bacteria, over three hundred years ago, using hand-ground lenses. Microscopy has come a long way since then. The following types of microscopes are in use today:

1. Optical or Light Microscope

This type of microscope is most commonly used in microbiology. It consists of two series of lenses**,** the **objective** and the **ocular lenses.** Together, they may magnify the organism up to 1,000 times. Light rays pass through a condenser to illuminate the object (specimen). The rays then pass through the lens of the objective to the eyepiece, and then to the observer's eye. **In a good light microscope, achromatic lenses are corrected to bring two wavelengths (typically red and blue) into focus on the same plane**. The **lens is designed to limit the effects of chromatic and spherical aberration.**

Total magnification = Magnification by objective lens × Magnification by eyepiece (in an oil-immersion lens magnification is 100x × 10x = 1,000 times)

Bacteria may be examined under the light microscope either in the living state as wet films (e.g., by the hanging drop method to visualise motility) or by fixation and staining to study the shape, size and arrangement (Fig. 2.2) of the organism.

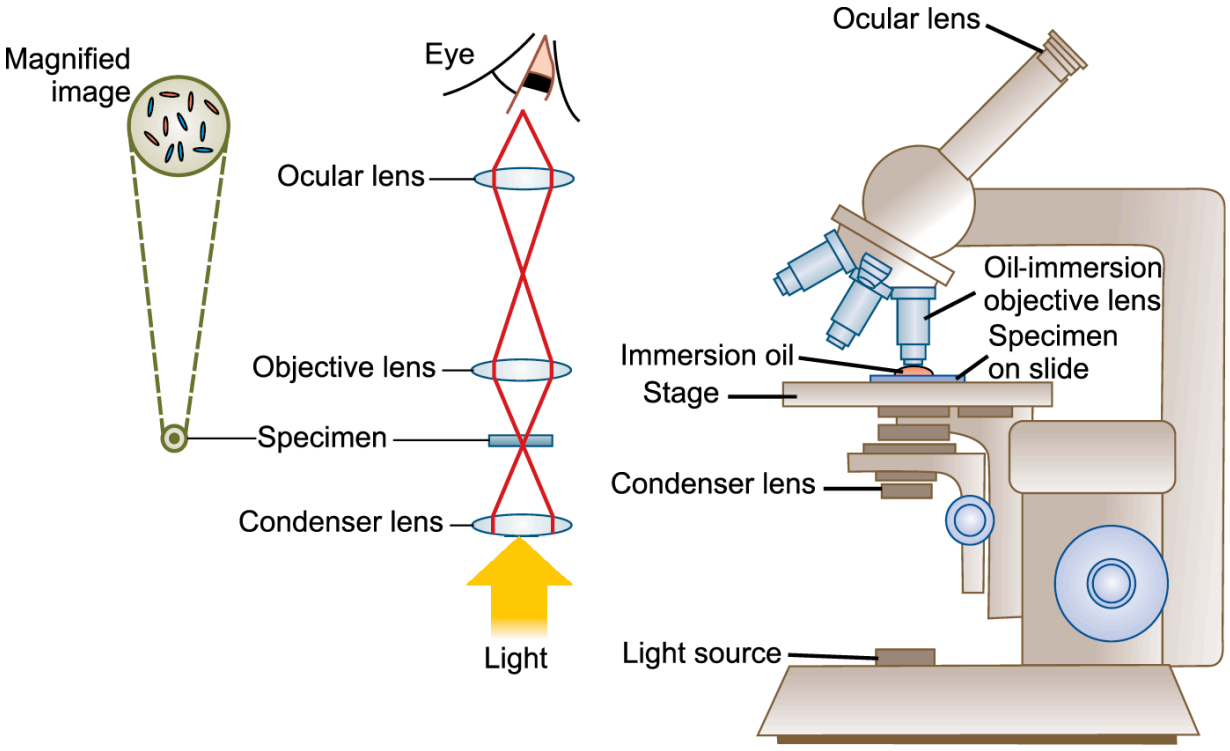


Fig. 2.2 Bright-field (light) microscope

Resolving power of a light microscope is the minimum distance between two closely placed objects that is required for the microscope to distinguish them as distinct objects. With a wavelength of 0.4 μm, the minimum separable diameter is 0.2 ∝m. With visible light and using the best optical systems, the limit of resolution of a light microscope is about 300 nm. If light of shorter wavelength is employed, as in the case of UV rays, the resolving power can be increased.

2. Phase-Contrast Microscope

This method of microscopy improves the contrast and makes evident the structures within cells, which differ in thickness or refractive index. The phase-contrast technique uses an optical mechanism to translate small variations in phase into corresponding changes in amplitude, which can be seen as differences in image contrast. These differences in refractive index between bacterial cells and the surrounding medium make them clearly visible. Retardation (by a fraction of wavelength) of rays of light that pass through the object, compared to those that pass through the surrounding medium, can be brought about by using a special ring. This produces 'phase' differences between the two types of rays (Fig. 2.3).

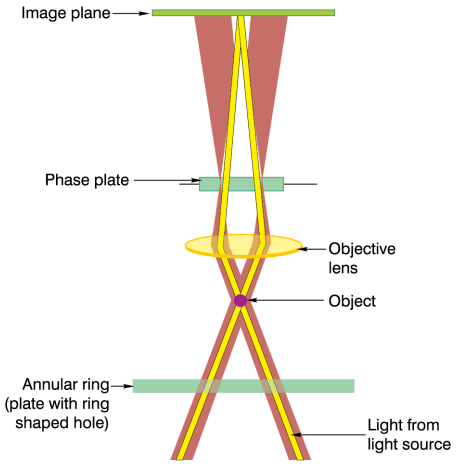


Fig. 2.3 Phase-contrast microscope

Living microorganisms, cells and tissues can be visualised as high-contrast images.

3. Fluorescence Microscope

In this microscope, light from a high-intensity source is used to excite a fluorescent agent, which in turn emits low-energy light of a longer wavelength, producing an image. Filters designed for that specific wavelength are used to block surrounding radiation, allowing the viewer to see only that which shows fluorescence. On exposure to excitation light, organisms are visually detected by the emission of fluorescent light by the dye with which they have been stained (Fig. 2.4). Microorganisms fluoresce following staining with fluorescent dyes called **fluorochromes, e.g., auramine O** and **rhodamine.** On the other hand, in immunofluorescence, antibodies are labelled with fluorescent dyes (like fluorescein isothiocynate or FITC) to form conjugates that are used to specifically stain a particular bacterial species (Fig. 2.5).

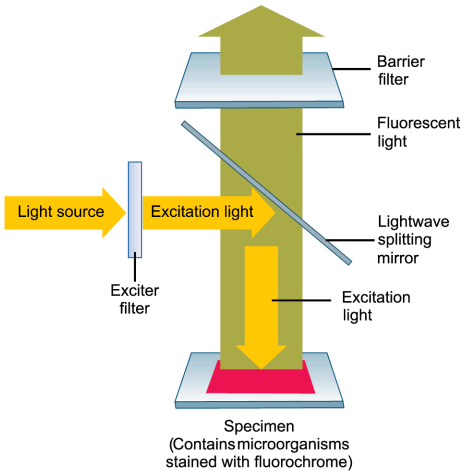


Fig. 2.4 Pathway of light in a fluorescence microscope

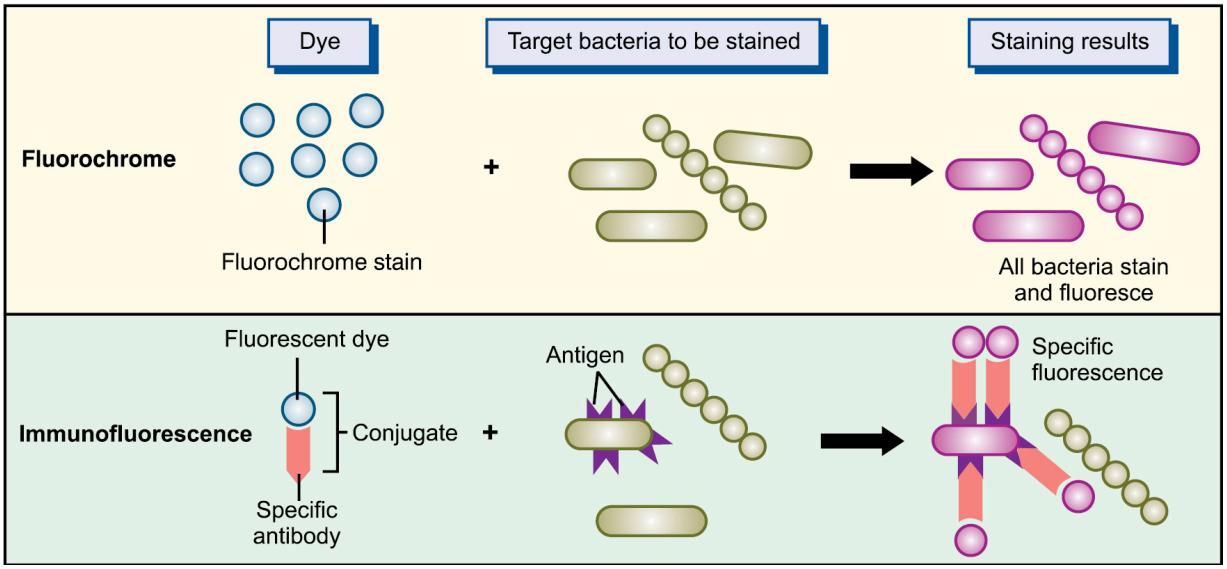


Fig. 2.5 Procedures of fluorochrome staining and immunofluorescence

4. Dark-Field/Dark-Ground Microscope

Another method of improving contrast is by using reflected light instead of transmitted light (as in the light microscope). This method is employed in the dark-field or dark-ground microscope. The essential part of the dark-field microscope is the **dark-field condenser** with a central circular stop. The specimen is illuminated by a cone of light without allowing any ray to fall directly on the objective lens. Light rays falling on the specimen are reflected or scattered on to the objective lens, whereby the object appears luminous against a dark background. The contrast gives an illusion of

increased resolution, and thus, very slender organisms such as spirochetes that are not visible under ordinary illumination can be clearly seen under the dark-field microscope (Fig. 2.6).

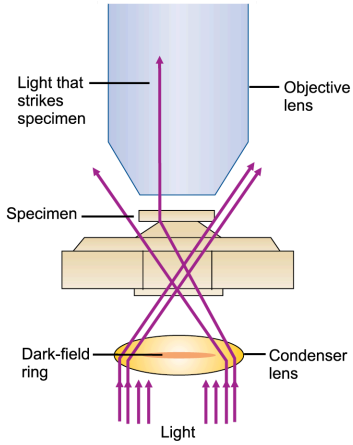


Fig. 2.6 Dark-field microscope

5. Differential Interference Contrast Microscope

This is a specialised type of microscope that not only reveals cell organelles but also enables quantitative measurement of the chemical constituents of cells such as lipids, proteins and nucleic acids.

6. Confocal Scanning Laser Microscope

The term 'confocal' is used when two lenses are arranged to focus on the same point. What is crucial to the principle of the confocal microscope is the presence of confocal pinholes which allow only the light from the plane of focus to reach the detector. In this type of microscopy, a light microscope is coupled with a laser light. The laser beam bounces off a mirror and is directed through a scanning device and then, through a pinhole. This excludes most of the light from the specimen, which is not from the microscope's focal plane. In a thick specimen, various layers can be seen by adjusting the plane of focus of the laser beam. Cells are stained with a fluorescent dye to make them more visible. The microscope is equipped with computer software to digitally process the images. Confocal scanning laser microscopes are used to examine tissue sections, in stem cell research and in fluorescence in-situ hybridisation.

7. Electron Microscope

Invented in 1931 by Max Knoll and Ernst Ruska, this microscope uses a beam of electrons instead of light rays (Fig. 2.7). The electron beam is focused by electromagnets, which are analogous to the lenses in the light microscope. The specimen, which is held in the path of the beam, scatters the electrons and produces an image. This image is focused on a fluorescent viewing screen (Fig. 2.8). Given that the wavelength of electrons is approximately 0.005 nm while that of visible light is 500 nm, the resolving power of the electron microscope should theoretically be 1,00,000 times that of the light microscope. However, in practice, the resolving power of the electron microscope is about 0.1 nm.

Since gas molecules scatter electrons, the specimen can be visualised only in a vacuum. This means that only dead and dried specimens can be examined with an electron microscope. This may lead to the distortion of cell morphology.

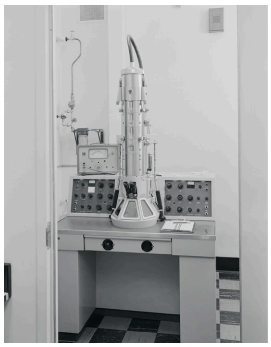


Fig. 2.7 Electron microscope (*Source:* Public Health Image Library, ID 12039 / C. Duncan / CDC)

Modifications of electron microscopy

- Shadow-casting with vapourised heavy metals has made taking pictures with good contrast and three-dimensional effect possible.
- Negative staining is another valuable technique in studying the fine structure of microorganisms and involves staining with phosphotungstic acid.
- Freeze-etching, a process involving the deep-freezing of specimens in liquid gas and the subsequent formation of carbon-platinum replicas of the material, prevents distortion of the image. Frozen cells may remain viable. Freeze-etching enables the study of cellular ultrastructure as it appears in the living state. The recent development of very-high-voltage electron microscopes may eventually render the examination of live specimens possible.
- Scanning electron microscope permits the study of cell surfaces with greater contrast and higher resolution than the shadow-casting technique.

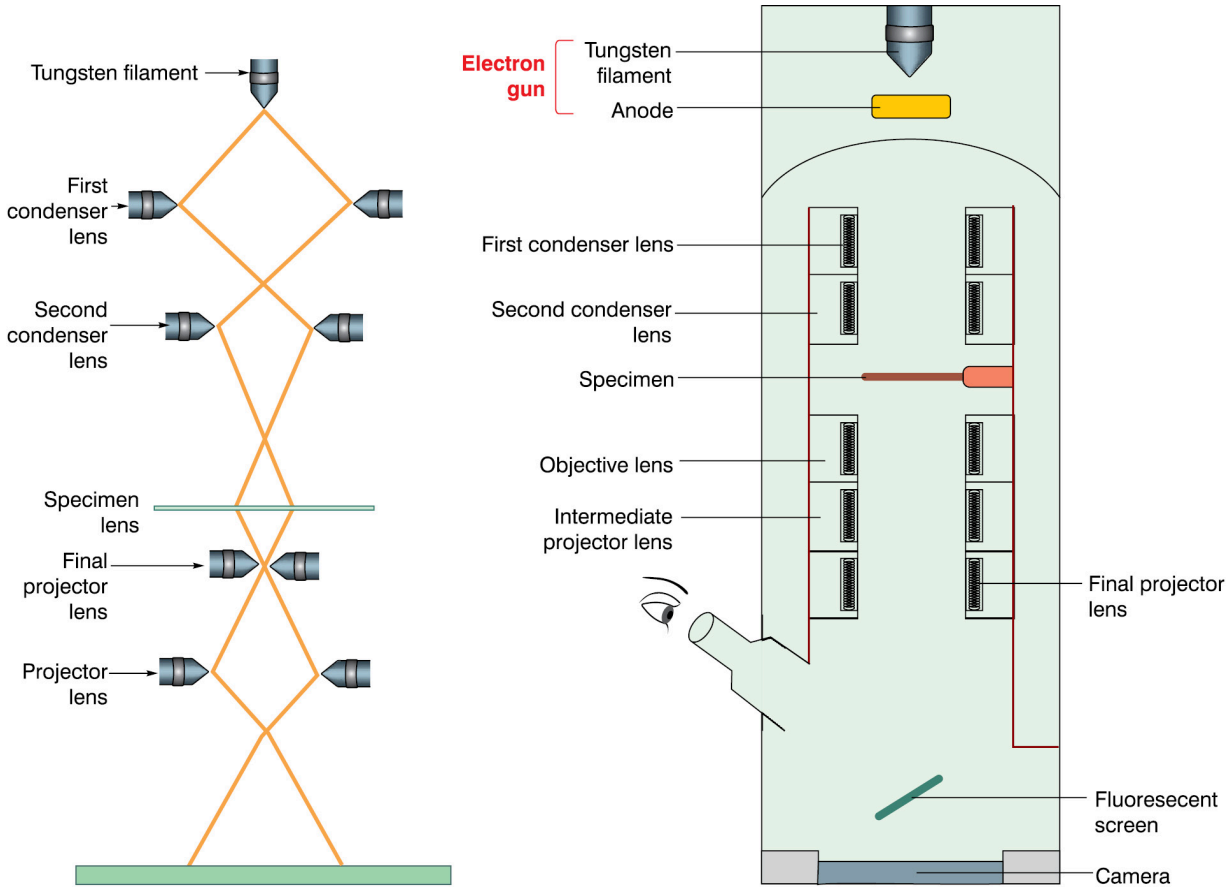


Fig. 2.8 Line diagram showing the path of electron beams and the electromagnets, which function as a series of condenser lenses in an electron microscope

MORPHOLOGY OF BACTERIA

BACTERIAL CELL STRUCTURE

Live bacteria do not show much structural detail under the light microscope due to the lack of contrast. Hence it is customary to stain bacteria for better visualisation.

Staining Techniques

Bacteria are stained with different dyes to produce a colour contrast. Routine methods of staining of bacteria involve drying and heat-fixing of smears. Heat-fixing causes the smear to adhere firmly to the slide, and allows the sample to readily take up stains. Bacteria have an affinity to basic dyes due to the acidic nature of their protoplasm. Some staining techniques commonly used in the bacteriology laboratory are:

1. ***Simple stains*** They provide colour contrast but impart the same colour to all bacteria. Two such stains are:

    - Methylene blue
    - Basic fuchsin

2. ***Negative staining*** This provides a dark-coloured background against which the unstained bacteria stand out in contrast. This is particularly useful in the **demonstration of bacterial capsules which do not take simple stains**. Very slender bacteria such as spirochetes, which are not demonstrable by simple staining, can be visualised by negative staining. The commonly used stains are:

    - India ink
    - Nigrosin

3. ***Impregnation methods*** Cells and structures that are too thin to be seen under the ordinary microscope may be rendered visible by the impregnation of the surface with silver to increase its thickness. This method is used to demonstrate spirochetes and bacterial flagella. One example is:

    - Levaditi's stain for spirochetes in tissues

4. ***Differential stains*** These stains impart different colours to different bacteria or bacterial structures. The two most widely used differential stains are:

    - Gram stain
    - Ziehl–Neelsen stain/**acid-fast stain**

Gram stain

Gram staining is a very important preliminary step in the initial characterisation and classification of bacteria.

It was originally devised by a Danish bacteriologist, **Hans Christian Joachim Gram** in ١٨٨٤. The procedure underwent several modifications

subsequently (Table 2.2).

Table 2.2 Modifications of Gram staining

Modification	Primary stain	Decolouriser/ Counterstain	Use
Weigert's modification	Carbol gentian violet	Aniline xylol	Useful for tissue sections
Preston and Morrell's modification	Crystal violet	Iodine acetone	Strengthens gram-positive staining
Jensen's modification	Methyl violet	Absolute alcohol/ neutral red	Better counterstain for gonococci and meningococci
Kopeloff and Beerman's modification	Methyl violet	Acetone/acetone alcohol	Useful for anaerobes and in bacterial vaginosis

Principle The exact mechanism of the Gram reaction is poorly understood. Various theories have been suggested:

- ***pH theory*** Gram-positive cells have a more acidic protoplasm, which may account for their retaining the primary basic dye more strongly than gram-negative bacteria.
- ***Cell wall theory*** The peptidoglycan of gram-positive bacteria is thick and thus able to retain the dye–iodine complex.
- The high **lipid content** of gram-negative bacteria makes them permeable to the secondary dye after decolourisation with organic solvents like acetone.

Decolourisation is not an all-or-none phenomenon. Gram-positive cells may be decolourised by prolonged treatment with the organic solvent. Conversely, inadequate decolourisation may cause all cells to appear gram-positive. **Gram-positive bacteria become gram-negative when their cell walls are damaged.**

Procedure

1. Thin smears are made on clean, grease-free slides and air-dried.
2. **Fixation** is done by passing the smears over a flame or by pouring cold methanol over the smear and allowing it to evaporate.
3. **Primary staining** is done with pararosaniline dyes such as crystal violet, methyl violet or gentian

violet.
4. Iodine solution (Gram's iodine) is used as a mordant.**Decolourisation** is brought about with an organic solvent such as ethanol, acetone or aniline.
5. **Counterstaining** is done with a dye of contrasting colour such as carbol fuchsin, safranine or neutral red.

Gram stain differentiates bacteria into two types:

- **Gram-positive** bacteria, which resist decolourisation and retain the primary stain, appearing violet
- **Gram-negative** bacteria, which are decolourised by organic solvents and take up the counterstain, appearing red (Fig. 2.9a and b)

Application Gram staining is essential for the identification of bacteria based on their:

- Growth requirements
- Susceptibility to antibiotics
- Pathogenicity

Ziehl–Neelsen stain (acid-fast stain)

Paul Ehrlich found that following staining with aniline dyes, tubercle bacilli resisted decolourisation with acids. The method commonly used today was modified from the original method developed by **Ziehl** and **Neelsen** (ZN stain).

Principle Acid-fastness is due to a variety of lipids, fatty acids and higher alcohols found in tubercle bacilli.

A lipid peculiar to acid-fast bacilli, a high-molecular-weight hydroxyl acid wax containing carboxyl groups (mycolic acid), is acid-fast in the free state.

Acid-fastness is not a property of lipids alone; it also depends on the integrity of the cell wall.

Procedure
1. The smear is first stained with the **primary stain**—a solution of strong carbol fuchsin with the application of heat.
2. It is then **decolourised** with 20% sulphuric acid.
3. It is further **counterstained** with a contrasting dye such as methylene blue.

4. Acid-fast bacteria retain the fuchsin (red) colour, while others take the counterstain.

Modified acid-fast stain

Cold method with Gabbet's methylene blue stain The smear is flooded with basic fuchsin-phenol stain and allowed to stand at room temperature for 10 minutes. It is then washed in running tap water and counterstained with Gabbet's methylene blue for 2 minutes. The smear is then washed in tap water and air-dried.

Acid-fast stain using 5% sulphuric acid *Mycobacterium leprae* resists decolourisation with 5% sulphuric acid (Fig. 2.9c and d).

Kinyoun's method *Nocardia* and *Legionella* resist decolourisation by 1% cold sulphuric acid, which is used as a decolouriser. The procedure is the same as that followed for ZN stain, except that instead of 20% or 5% sulphuric acid, cold 1% acid is used as the decolourising agent. The bacteria appear pink with a blue background.

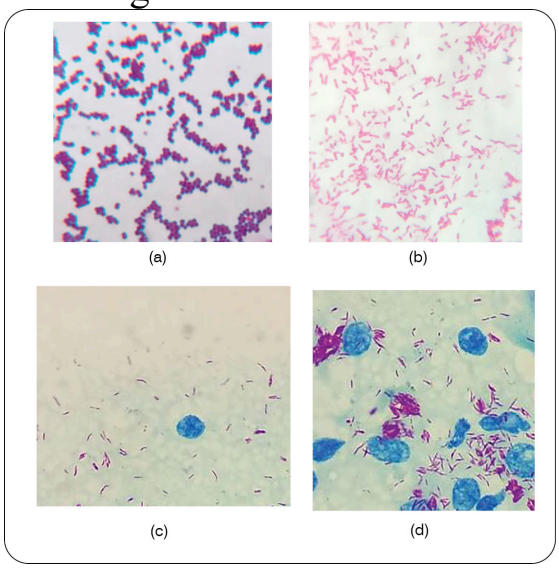


Fig. 2.9 Colour images of bacteria stained using different stains: (a) gram-positive cocci; (b) gram-negative bacilli; (c) Ziehl–Neelsen acid-fast *M. tuberculosis* and (d) acid-fast *M. leprae* (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Stains for demonstrating intracellular granules

Some special stains are required to demonstrate intracellular granules that are present in some bacteria.

Albert's stain

Principle On staining with Albert's stain, the granules of ***Corynebacterium diphtheriae*** take up a bluish purple colour. Hence they are called metachromatic granules.

Procedure
1. The smear is stained with Albert I stain and then drained and washed.
2. Albert II is poured over the smear and then drained.

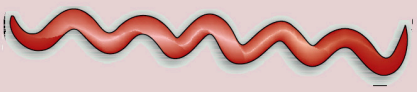
Application This method is used to demonstrate *Corynebacterium diphtheriae* in throat swabs or cultures. Other stains that can demonstrate granules are **Neisser's** and **Ponder's** stains.

SHAPE OF BACTERIA

Depending on their shape, bacteria are classified into several types (Table 2.3).

Some species of bacteria exhibit great variation in the shape and size of individual cells. This is known as **pleomorphism.** Pleomorphic and involuted forms are often the result of defective cell wall synthesis.

Table 2.3 Classification of bacteria based on shape

Type of bacteria	Example	Diagrammatic representation
Cocci (from *kokkos*, meaning berry)	*Staphylococcus, Streptococcus*	
Bacilli (rod-shaped) (from *baculus*, meaning rod)	*E. coli, Proteus, Salmonella*	
Vibrio (comma-shaped)	*Vibrio cholerae*	
Spirilla	*Spirillum minus*	
Spirochetes (from *speira*, meaning coil and *chaite*, meaning hair)	*Borrrelia, Leptospira, Treponema*	
Filamentous bacteria	Actinomycetes	
Amorphous morphology: round or oval bodies and interlacing filaments	Mycoplasma	

ARRANGEMENT OF BACTERIA

The arrangement of groups of bacteria is determined by the plane through

which binary fission takes place and by the tendency of the daughter cells to remain attached even after division.

In culture or tissues, cocci may be arranged in clusters, chains, pairs or tetrads while bacilli form chains which are end-to-end or at angles to each other (Table 2.4).

Table 2.4 Arrangement of bacteria

Type of arrangement	Example	Diagrammatic representation
Cocci		
Clusters	Staphylococci	
Chains	Streptococci	
Pairs	Pneumococci	
Tetrads	Micrococci	
Bacilli		
Chains are end to end	Bacilli	
Rods are at angles to each other, presenting a cuneiform or Chinese letter pattern	Corynebacteria	

BACTERIAL ANATOMY

A bacterial cell (Fig. 2.10) is composed of the **outer layer** or **cell envelope, the cell interior** and **additional structures.**

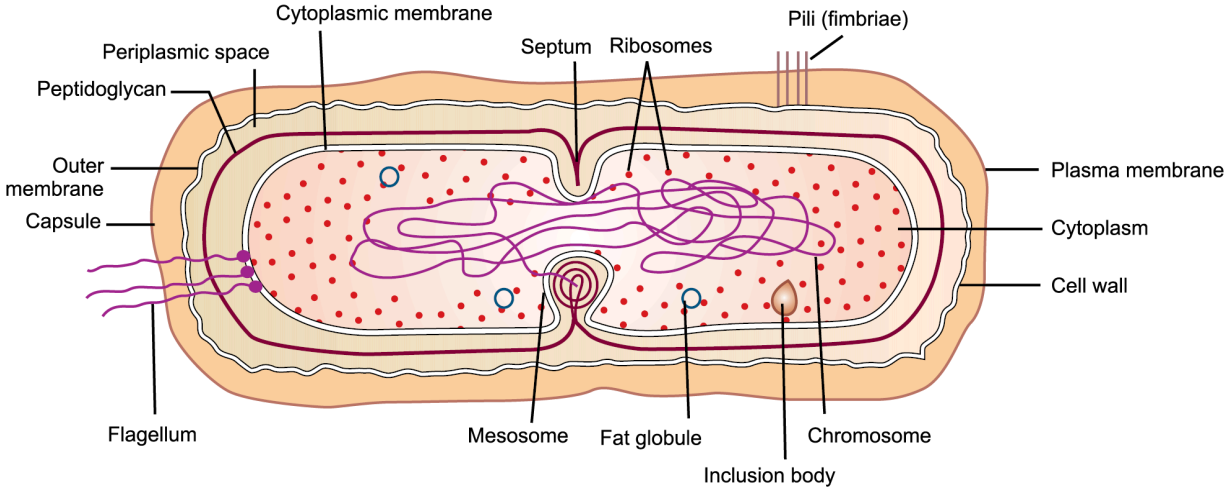


Fig. 2.10 Diagram of a bacterial cell

CELL ENVELOPE

The cell envelope consists of two components:

- A **rigid cell wall**
- A **cytoplasmic** or **plasma membrane** (beneath the cell wall)

Cell Wall

The cell wall confers shape, rigidity and plasticity to the cell. It does not stain with simple stains and hence cannot be visualised by light microscopy.

Demonstration

The cell wall can be demonstrated by:

- Plasmolysis (the cytoplasm shrinks in a hypertonic solution, whereas the cell wall remains intact)
- Microdissection
- Reaction with specific antibodies
- Mechanical rupture of the cell
- Differential staining procedures
- Electron microscopy

Structure

Bacterial cell walls are about 10–25 nm thick and account for about 20–30% of the dry weight of the cell. Chemically, the cell wall is composed of mucopeptide (**peptidoglycan** or **murein**) scaffolding formed by ***N*-acetyl glucosamine** and ***N*-acetyl muramic acid** molecules alternating in chains, which are cross-linked by peptide chains (Fig. 2.11). The interstices of this scaffolding contain other chemicals, varying in the different species.

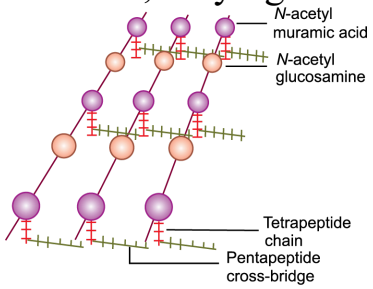


Fig. 2.11 Chemical structure of bacterial cell wall

In general, the walls of gram-positive bacteria have a simpler chemical nature than those of gram-negative bacteria (Table 2.5). The cell wall carries bacterial antigens that are important in virulence and immunity.

Table 2.5 Comparison of cell walls of gram-positive and gram-negative bacteria

Characteristic	Gram-positive bacteria	Gram-negative bacteria
Thickness (peptidoglycan)	Thicker	Thinner
Variety of amino acids	Few	Several
Aromatic and sulphur-containing amino acids	Absent	Present
LPS and outer membrane	Absent	Present
Teichoic acid	Present	Absent

Cell wall of gram-positive bacteria

Peptidoglycan layer It is thicker (15–80 nm) than that of gram-negative bacteria (2–3 nm).

Teichoic acid A polymer of ribotol or glycerol, teichoic acid is a major surface antigen found in abundance in the cell wall of gram-positive bacteria. Two **types** of teichoic acid are present:

- Cell wall teichoic acid—linked to peptidoglycan
- Membrane teichoic acid (lipoteichoic acid)—linked to membrane glycolipid (Fig. 2.12a)

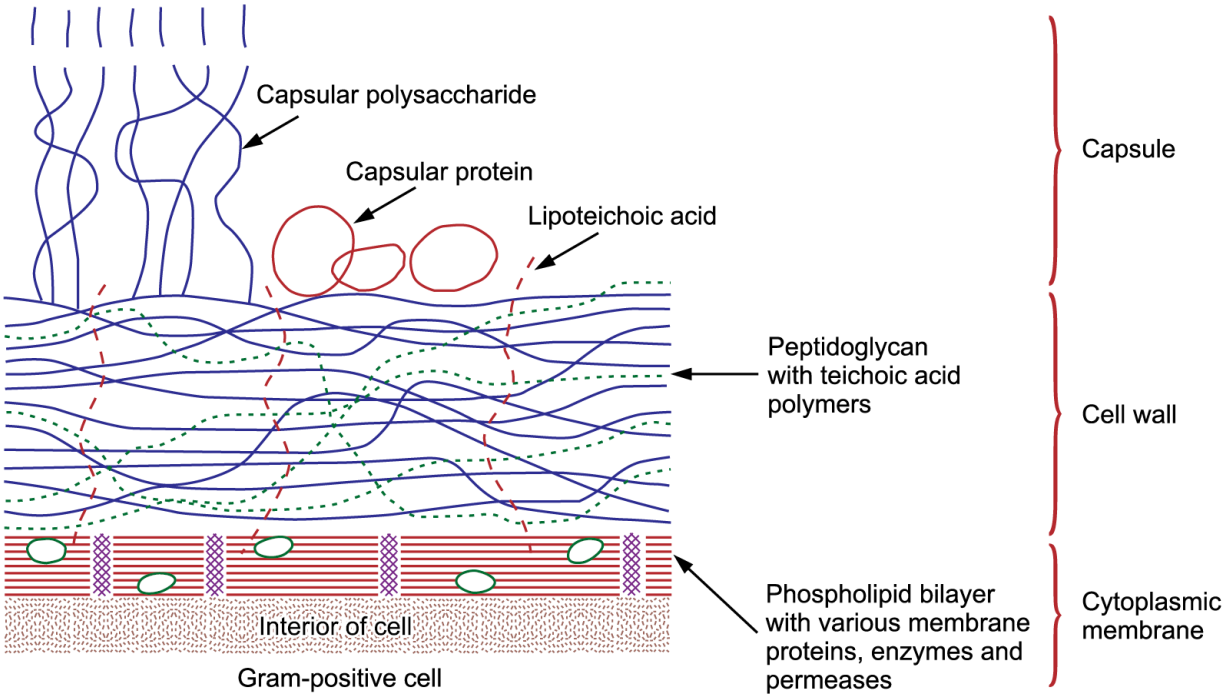


Fig. 2.12 (a) Gram-positive

Cell wall of gram-negative bacteria

Lipopolysaccharides (LPS) These are present on the cell walls of gram-negative bacteria and account for their **endotoxic activity** and O antigen specificity. They were formerly known as the Boivin antigen. The **LPS** layer consists of **three regions**:

- **Region I** is the O polysaccharide portion, which determines O antigen specificity.
- **Region II** is the core polysaccharide.
- **Region III** is the glycolipid portion (**lipid A**) and is responsible for the **endotoxic** activities of the organism, that is, pyrogenicity, lethal effect, tissue necrosis, anticomplementary activity, B cell mitogenicity, immunoadjuvant property and antitumour activity.

Outer membrane The outermost layer of the gram-negative bacterial cell wall is called the outer membrane. It contains various proteins known as **outer membrane proteins (OMP)**. Among these are **porins**, which form transmembrane pores that serve as diffusion channels for small molecules. They also serve as specific receptors for some bacteriophages.

Lipoprotein This attaches proteins of the peptidoglycan layer to lipids of the outer membrane.

Peptidoglycan This is a thin layer (2–3 nm) that is bound by the lipoprotein and plasma membrane (Fig. 2.12b).

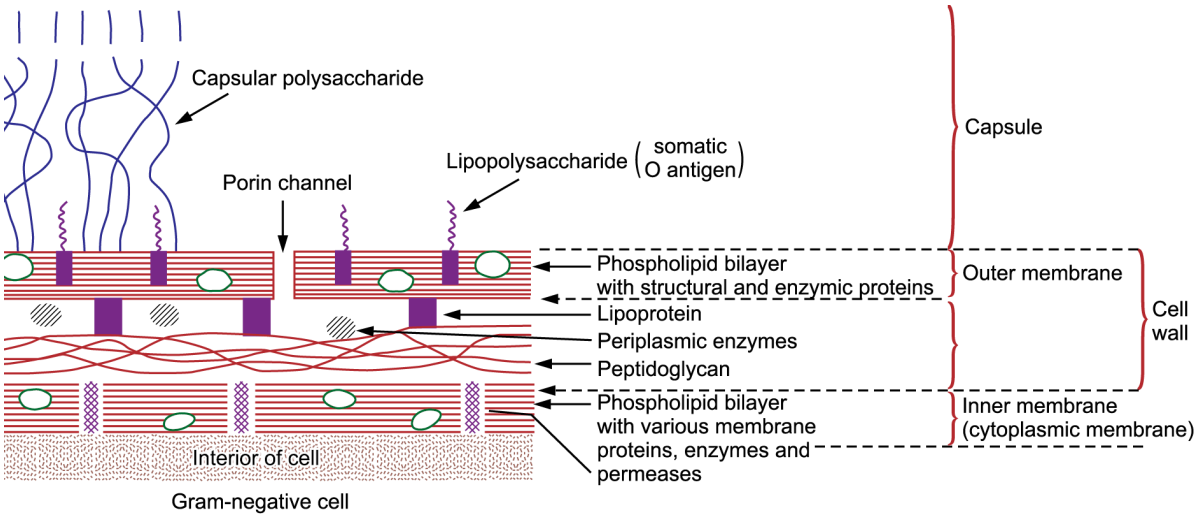


Fig. 2.12 (b) gram-negative cell wall

Inhibition of cell wall synthesis

An enzyme, **lysozyme,** normally present in many tissue fluids, lyses susceptible bacteria by splitting the cell wall mucopeptide links. This can result in:

Protoplasts When lysozyme acts on a gram-positive bacterium in a hypertonic solution, a protoplast is formed, consisting of the cytoplasmic membrane and its contents.

Spheroplasts When lysozyme acts on gram-negative bacteria, the result is a spheroplast which differs from the protoplast in that some cell wall material is retained. Protoplasts and spheroplasts are spherical, regardless of the original shape of the bacterium.

L-forms

Klieneberger-Nobel, while studying cultures of *Streptobacillus moniliformis*, observed swollen cells and other aberrant morphological forms. She named them L-forms, after Lister Institute, London, where the observation was made. L-forms are seen in several species of bacteria, developing either spontaneously or in the presence of penicillin or other

agents that interfere with cell wall synthesis. These cells were devoid of cell walls. L-forms may be either **unstable** (when the morphological abnormality is maintained only in the presence of penicillin or other inducing agents) or **stable** (when the aberrant form becomes the permanent feature of the strain and is retained in serial subcultures). L-forms resemble mycoplasma in several ways, including morphology, type of growth on agar and filterability. It is possible that mycoplasmas represent stable L-forms of as yet unidentified parent bacteria.

Cell wall-deficient bacteria may have a role in chronic persistent infections.

Cytoplasmic Membrane

The cytoplasmic (plasma) membrane is a thin (5–10 nm) layer lining the inner surface of the cell wall and separating it from the cytoplasm. It acts as a semipermeable membrane, actively controlling the flow of metabolites to and from the protoplasm. Passage through the membrane is not solely a function of the molecular size of the particles but also depends on the presence of specific enzymes (**permeases**) in the membrane. Electron microscopy shows the presence of three layers constituting a **unit membrane** structure. Chemically, the membrane consists of lipoproteins with small amounts of carbohydrates. Sterols are absent, except in mycoplasma.

CELL INTERIOR

The interior of a bacterial cell consists of the **protoplasm**, comprising the cytoplasm, cytoplasmic inclusions such as ribosomes and mesosomes, granules, vacuoles and the nuclear body.

Cytoplasm

The bacterial cytoplasm is a colloidal system of a variety of organic and inorganic solutes in a viscous, watery solution. It contains ribosomes, mesosomes, inclusions and vacuoles. Unlike eukaryotic cytoplasm, it does not exhibit internal mobility (**protoplasmic streaming**) and does not possess endoplasmic reticulum or mitochondria. The cytoplasm stains uniformly with basic dyes in young cultures but becomes increasingly

granular with age.

Intracytoplasmic Inclusions

Bacterial cells contain certain intracytoplasmic materials that play a vital role in protein synthesis and the metabolism of the cells. They are the following.

Ribosomes

The most essential of intracytoplasmic material, ribosomes are the **centres of protein synthesis**. They are slightly smaller than the ribosomes of eukaryotic cells (sedimentation constant 70S) and are seen integrated with linear strands of mRNA to form polysomes.

Mesosomes (chondroids)

These are vesicular, convoluted or multi-laminated structures formed as invaginations of the plasma membrane into the cytoplasm. They are more prominent in gram-positive bacteria. They are the **principal sites of respiratory enzymes** in bacteria and are analogous to the mitochondria of eukaryotes. Mesosomes are often seen in relation to the nuclear body and the site of synthesis of cross-wall septa, suggesting that they coordinate nuclear and cytoplasmic division during binary fission.

Metachromatic (volutin) granules

Found in some bacterial cells, these granules are considered to be reserves of energy for metabolism. They are volutin (polymetaphosphate in diphtheria bacilli), polysaccharide, lipid or crystal in nature. These inclusions differ in different species and depend on the age and condition of the culture. They can be demonstrated by special stains like Albert's and Neisser's stains.

Nuclear Material

Bacteria, being prokaryotic cells, do not have a membrane-bound nucleus. Instead, their deoxyribonucleic acid (DNA) is found in the nucleoid. It can be demonstrated by acid or ribonuclease hydrolysis and subsequent staining for nuclear material. The nucleoid may be seen by electron microscopy. Nuclear bodies appear as oval or elongated bodies, generally one per cell.

Some cells may possess two or more nuclear bodies due to asynchrony between nuclear and cytoplasmic division.

The nuclear DNA is not associated with basic protein. The genome consists of a single molecule of double-stranded DNA arranged in the form of a circle, which may open under certain conditions to form a long chain, about 1 μm in length. The bacterial chromosome is haploid and replicates by simple fission unlike the chromosome of other cells, which replicates by mitosis. The differences between the nuclei of bacteria and those of other organisms form the basis for classifying them as prokaryotes and eukaryotes (Table 2.1).

Bacteria may possess **extranuclear genetic elements termed plasmids** or **episomes**. Besides being transmitted to daughter cells during binary fission, these may be transferred from one bacterium to another either through conjugation or by bacteriophages. They confer toxigenicity and drug resistance to the bacteria.

ADDITIONAL STRUCTURES

The cell may be enclosed in a viscid layer, which may either be a loose slime layer **(glycocalyx)** or a tightly organised **capsule**. Some bacteria have filamentous appendages that protrude from the cell surface—the **flagella** (organs of locomotion) and the **fimbriae** (organs for adhesion) (Fig. 2.10).

Slime Layer and Capsule

Many bacteria secrete a viscid material around the cell surface. When this is organised into a sharply defined structure, as in *S. pneumoniae*, it is known as the **capsule**. When it is a loose, undemarcated secretion, as in *Leuconostoc*, it is called the **slime layer**. Capsules too thin to be seen under the light microscope are called **microcapsules**. The slime may be polysaccharide (*S. pneumoniae*) or polypeptide (anthrax bacillus) in nature. Some bacteria may have both a capsule and a slime layer (e.g., *Streptococcus salivarius*). Bacteria that secrete large amounts of slime produce mucoid growth on agar (e.g., *Klebsiella pneumoniae*).

Demonstration of capsule

The capsule of bacteria may be demonstrated using staining or serological methods.

Staining

Slime has little affinity for basic dyes and is not visible in Gram-stained smears. **Special capsule staining** techniques are available, usually employing copper salts as mordants. Capsules may be readily demonstrated by **negative staining in wet films with India ink** and are seen as clear halos around the bacteria, against a black background (Fig. 2.13). India ink is often used to demonstrate capsulated microorganisms from clinical material, for example, *S. pnuemoniae* or *Cryptococcus* in CSF of patients with meningitis.

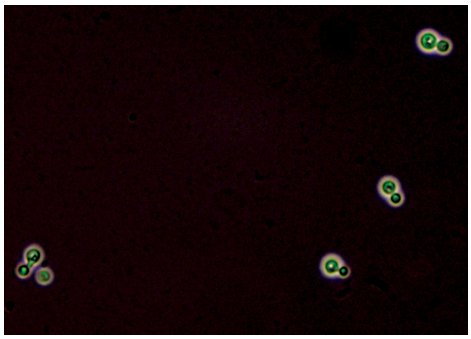


Fig. 2.13 Cryptococcus capsule seen (as a halo) by India ink staining
(*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Serological methods

When a suspension of a capsulated bacterium is mixed with its specific anticapsular serum, the capsule appears very prominent and 'swollen' due to an increase in its refractivity. This **capsule swelling or Quellung reaction** (Neufeld, 1902) has been widely employed for the typing of *S. pneumoniae*. Capsules protect bacteria from lytic enzymes. They also contribute to the virulence of pathogenic bacteria by inhibiting phagocytosis. Repeated subcultures in vitro lead to loss of the capsule and also virulence.

Flagella

The organelles of locomotion, flagella, are either single or multiple, unbranched, long and sinuous filaments.

Structure

Each flagellum consists of three distinct parts, the filament, the hook and

the basal body.

- The **filament** protrudes out of the cell and is connected to the hook at the cell surface.
- The **hook** is attached to the basal body.
- The **basal body** is embedded in the cell envelope. The hook and basal body are antigenically different.

Mechanical detachment of the filament does not impair the viability of the cell. Flagella are 3–20 ∝m long, are of uniform diameter (0.01–0.013 ∝m) and terminate in a square tip. Though wavelength and thickness of the filament are characteristic of each species, some bacteria exhibit **biplicity**, that is, they have flagella of two different wavelengths. Flagella are made up of a protein (**flagellin**) similar to keratin or myosin. Though flagella of different genera of bacteria have the same chemical composition, they are antigenically different. Flagellar antigens (known as H antigens) induce specific antibodies in high titres. Flagellar antibodies are not protective but are useful in serodiagnosis (Fig. 2.14).

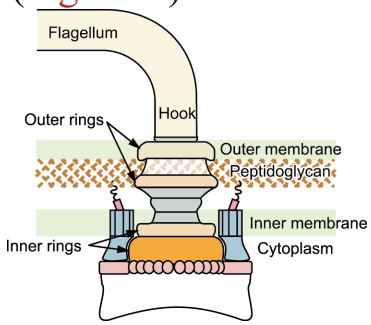


Fig. 2.14 General structure of the flagellum of a gram-negative bacterium

Arrangement

The presence or absence of flagella and their number and arrangement (Fig. 2.15) differ in different bacteria. There are two main types of flagellar arrangements:

- **Peritrichous** (all round the cell), as in typhoid bacilli
- **Polar** (situated at one or both ends of the cell), which may be further arranged as follows:
  a) Single (**monotrichous**), as in cholera vibrios

b) In tufts **(lophotrichous)**, as in spirilla
c) At both poles (**amphitrichous**)

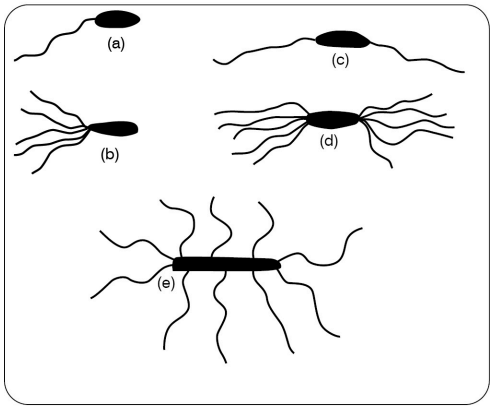


Fig. 2.15 Types of flagellar arrangement: (a) monotrichous; (b) lophotrichous; (c) amphitrichous; (d) amphilophotrichous and (e) peritrichous

Demonstration

Flagella are less than 0.02 μm in thickness and hence beyond the limit of resolution of the light microscope. They may, in some instances, be seen under **dark -ground illumination**. They can be visualised by **special staining techniques** in which their thickness is increased by mordanting (e.g., by silver impregnation), or by **electron microscopy**.

Motility

Due to the difficulty of demonstrating flagella directly, their presence is usually inferred from the motility of bacteria.

Under the microscope, active motility has to be differentiated from the passive movements of the cells either due to air currents or due to Brownian movement. Bacterial motility may range from the slow '**stately' motion** of peritrichate bacteria (e.g., *Bacillus*) to the **darting movement** of polar flagellated vibrios (200 mm per second).

Motility can also be observed by noting the spreading type of growth on a semisolid agar medium.

Fimbriae

Some gram-negative bacilli carry very fine, hair-like surface appendages called fimbriae or **pili.** They are shorter and thinner than flagella (about 0.5 μm long and less than 10 nm thick) and project from the cell surface as

straight filaments. At least eight morphological types of pili are known, classifiable as either **common** or **sex pili** on the basis of their function. Pili comprise self-aggregating monomers of pilin. They originate in the cell membrane. Fimbriae can be seen only under the electron microscope. They are best developed in freshly isolated strains and in liquid cultures. They tend to disappear following subculture on solid media.

Fimbriae function as **organs of adhesion**, helping the cells to adhere firmly to particles of various kinds. This property may serve to anchor the bacteria in nutritionally favourable microenvironments.

Fimbriated bacteria form **surface pellicles** in liquid media.

Many fimbriated cells (e.g., *Escherichia* and *Klebsiella*) **agglutinate red blood cells** of guinea pigs, fowl, horses and pigs strongly, human and sheep cells weakly, and ox cells scarcely. **Hemagglutination** provides a simple method of detecting the presence of such fimbriae. It is specifically inhibited by D-mannose (mannose-sensitive).

Fimbriae are antigenic. As members of different genera may possess the same fimbrial antigen, it is necessary to ensure that the bacterial antigens employed for serological tests and preparation of antisera are devoid of fimbriae.

A special type of fimbriae are the sex pili. These are longer and fewer in number than other fimbriae. They are found on '**male**' **(donor)** bacteria and help in the attachment of those cells to '**female**' **(recipient)** bacteria, forming hollow conjugation tubes through which, it is assumed, genetic material is transferred from the donor to the recipient cell.

Pili are classified into different **types** (e.g., F and I) based on susceptibility to specific bacteriophages.

Spore

Some bacteria possess the ability to form highly resistant resting stages called spores (e.g., *Bacillus* and *Clostridium*). Each bacterium forms one spore, which on germination, forms a single vegetative cell. Sporulation in bacteria, therefore, is not a method of reproduction. As bacterial spores are formed inside the parent cell, they are called **endospores**.

While the exact stimulus for sporulation is not known, it has been observed that it occurs after a period of vegetative growth and is presumed

to be related to the depletion of exogenous nutrients.

Process of sporulation and structure of the spore

- **Forespore** Sporulation is initiated by the appearance of a clear area, usually near one end of the cell, which gradually becomes more opaque to form the 'forespore'.
- **Spore wall** The fully developed spore has the nuclear body at its core, surrounded by the spore wall, a delicate membrane from which the cell wall of the future vegetative bacterium will develop.
- **Spore cortex** Outside the spore wall is the thick spore cortex.
- **Spore coat** This is a multilayered tough coat that encloses the spore cortex.
- **Exosporium** Some spores have an additional outer covering called exosporium which may have distinctive ridges and grooves (Fig. 2.16).

New antigens appear on sporulation.

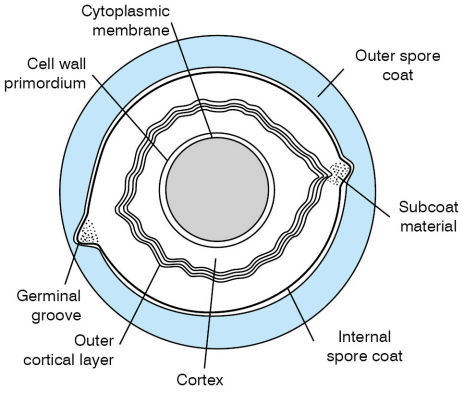


Fig. 2.16 Diagrammatic representation of a bacterial spore

Shape and position

Young spores are seen attached to the parent cell. The shape and position of the spore and its size relative to the parent cell are species characteristics. Spores may be **central** (equatorial), **terminal** or **subterminal**. They may be **oval** or **spherical**. They may or may not distend the bacillary body (Figs. 2.17 and 2.18).

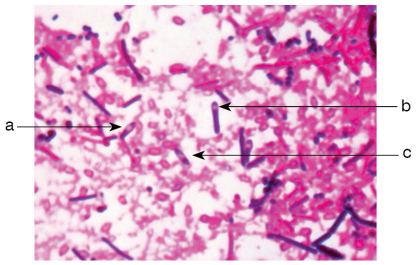


Fig. 2.17 Spores seen in unstained mixed clostridial infection: (a – terminal, b – subterminal and c – central) (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

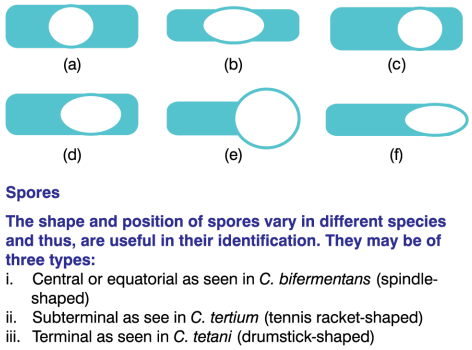

(a)
(b)
(c)
(d)
(e)
(f)
Spores
The shape and position of spores vary in different species and thus, are useful in their identification. They may be of three types:
i. Central or equatorial as seen in C. bifermentans (spindle-shaped)
ii. Subterminal as see in C. tertium (tennis racket-shaped)
iii. Terminal as seen in C. tetani (drumstick-shaped)

Fig. 2.18 Types of bacterial spores: (a) spherical central; (b) oval central; (c) spherical subterminal; (d) oval subterminal; (e) spherical terminal and (f) oval terminal

Resistance

Bacterial spores constitute some of the most resistant forms of life. They may remain viable for centuries under unfavourable conditions. They are extremely resistant to desiccation and relatively so to chemicals and heat. Though some spores may resist boiling for prolonged periods, spores of all medically important species are destroyed by autoclaving at 120°C for 15 minutes. Methods of sterilisation and disinfection should ensure that spores are also destroyed.

When conditions become conducive for growth, spores germinate. The spore loses its refractivity and swells. The spore wall is shed and the germ cell appears by rupturing the spore coat and elongates to form the vegetative bacterium.

Demonstration

Spores may be seen in unstained preparations as refractile bodies. The forespore stains intensely but once the spore envelope is formed, the spore

does not stain readily. Spores appear as unstained areas in Gram-stained preparations, but are acid-fast, hence they can be stained by a **modification of the Ziehl–Neelsen technique**.

Involution forms may develop due to ageing cultures and autolytic enzymes.

PHYSIOLOGY OF BACTERIA

GROWTH AND MULTIPLICATION OF BACTERIA

Cell Division

Bacteria divide by **binary fission** (Fig. 2.19). When a bacterial cell reaches a certain size, it divides to form two daughter cells. Nuclear division precedes cell division and, therefore, in a growing population, many cells carrying two nuclear bodies can be seen. The cell divides by a constrictive or pinching process or by the in-growth of a transverse septum across the cell. In some species, the daughter cells may remain partially attached after division.

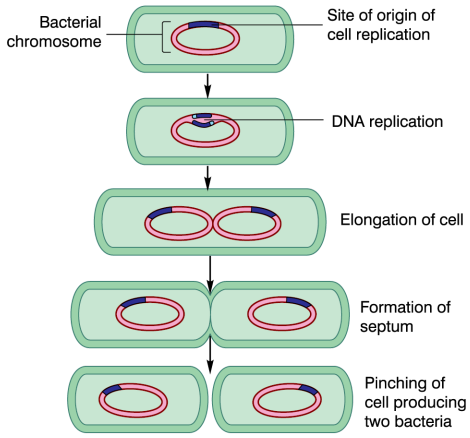


Fig. 2.19 Bacterial cell division by binary fission

Generation time

The time required for a bacterium to give rise to two daughter cells under optimum conditions is known as its generation time or **population doubling time**. In coliform bacilli and many other medically important bacteria, the generation time is about 20 minutes. Some bacteria are slow-

growing—the generation time of tubercle bacilli is about 20 hours and that of lepra bacilli is as much as about 20 days. As bacteria reproduce rapidly and by geometric progression, a single bacterial cell can theoretically give rise to 10^{21} progeny in 24 hours, with a mass of approximately 4,000 tonnes!

Growth

Bacteria need to be cultivated in culture media to study them and understand their growth, metabolites and other physical characterestics.

Batch culture In practice, when bacteria are grown in liquid medium (batch culture), multiplication is arrested after a few cell divisions due to the depletion of nutrients or the accumulation of toxic products.

Continuous culture By replenishing nutrients and removing bacterial cells **(in a chemostat or turbidistat)**, it is possible to maintain a continuous culture of bacteria for industrial or research purposes.

Though the human body is an inexhaustible source of nutrients, bacteria have to contend with the host's defence mechanism, which arrests their growth. Bacteria growing on solid media form colonies. Each colony represents a clone of cells derived from a single parent cell. In liquid media, growth is diffuse.

Bacterial growth curve

When a bacterium is seeded into a suitable liquid medium and incubated, its growth follows a definite course. If bacterial counts are made at regular intervals after inoculation and plotted in relation to time, a growth curve is obtained. The various stages of the bacterial growth curve are associated with **morphological** and **physiological** alterations of the cells (Fig. 2.20). The phases of the growth curve are as follows:

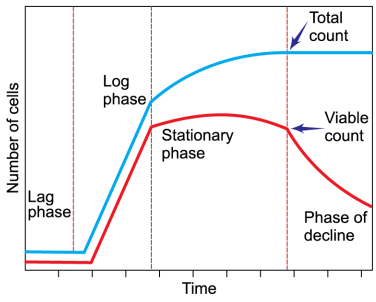


Fig. 2.20 Bacterial growth curve: The viable count shows the lag, log, stationary and decline phases; in the total count, the phase of decline is not evident

Lag phase

Immediately following the seeding of a culture medium, there is no increase in the number of cells, though there may be an increase in the size of the cells. This initial period is the time required for the organisms to adapt to the new environment, during which, necessary enzymes and metabolic intermediates are built up for multiplication. The duration of the lag phase varies with the species, size of the inoculum, nature of the culture medium and environmental factors such as temperature. **Maximum size is obtained at the end of the lag phase.**

Log (logarithmic) or exponential phase

Following the lag phase, the cells start dividing and their numbers increase exponentially or by geometric progression with time. When viable count is plotted against time, a straight line is obtained. Cells are smaller and stain uniformly. **They are more susceptible to the action of antibiotics during this phase.**

Stationary phase

After a varying period of exponential growth, cell division stops due to the depletion of nutrients and accumulation of toxic products. The number of progeny cells is just enough to replace the dying cells. The viable count remains stationary as equilibrium exists between the dying and new cells. Cells which are frequently gram-variable have intracellular storage granules. **Sporulation occurs at this stage**. Some organisms produce secondary metabolic products such as exotoxins and antibiotics.

Phase of decline

The population decreases due to cell death. Besides nutritional depletion and toxic accumulation, autolytic enzymes may also cause cell death.

The total count parallels the viable count up to the stationary phase, after which it continues steadily without any phase of decline. However, autolytic bacteria show a phase of decline of total count also. **Involution forms are common.**

Bacterial counts

Growth in terms of numbers can be studied by bacterial counts. Two types of bacterial counts can be made—total and viable.

The **total count** gives the total number of cells in the sample, irrespective of whether they are living or not. It can be obtained by:

- Direct counting under the microscope using counting chambers
- Counting in an electronic device as in the Coulter counter

The **viable count** measures the number of living cells in a culture. Viable counts are obtained by either the **dilution method or the plating method**.

In the **dilution method**, the suspension is diluted to a point beyond which unit quantities do not yield growth when inoculated into a suitable liquid media (extinction). This method does not give accurate values but is used widely in water bacteriology for the estimation of the '**presumptive coliform count**' in drinking water.

In the **plating method**, appropriate dilutions are inoculated on solid media, either on the surface of plates or as pour plates. The number of colonies that develop after incubation gives an estimate of the viable count.

BACTERIAL NUTRITION

The principal constituent of bacterial cells is water, which represents about 80 per cent of the total weight. Proteins, polysaccharides, lipids, nucleic acids, mucopeptides and low-molecular-weight compounds make up the rest.

Nutritional Requirements of Bacteria

For growth and multiplication of bacteria, the minimum nutritional requirements are **water, a source of carbon, a source of nitrogen and some inorganic salts.** Some bacteria require **organic compounds** in minute quantities as growth factors. In many cases, growth factors (or **bacterial vitamins**) are identical to the vitamins necessary for mammalian nutrition, e.g., thiamine, riboflavin, nicotinic acid, pyridoxine, folic acid and vitamin B_{12}. Bacteria also require phosphate and sulphate and the cations sodium, potassium, magnesium, iron, manganese and calcium.

All bacteria require small amounts of carbon dioxide for growth. This

requirement is usually met by the carbon dioxide present in the atmosphere or that produced endogenously by cellular metabolism. **Capnophilic** bacteria like *Campylobacter* and *Brucella abortus* require much higher levels of carbon dioxide.

Classification of Bacteria Based on Various Nutritional Requirements

Classification based on energy requirements

Based on their **energy requirements**, bacteria can be classified as:

- ***Phototrophs*** Bacteria that derive energy from sunlight
- ***Chemotrophs*** Bacteria that derive energy from chemical reactions
- ***Autotrophs*** Bacteria that synthesise their own organic material
- ***Heterotrophs*** Bacteria that cannot synthesise their metabolites; they utilise atmospheric carbon dioxide and nitrogen

Classification based on oxygen requirements

Based on the requirement of oxygen for growth and viability, bacteria are divided into the following types:

i) **Aerobic bacteria** require oxygen and may be of two types:

- **Obligate aerobes** like *Vibrio cholerae,* which will grow only in the presence of oxygen
- **Facultative anaerobes,** which are ordinarily aerobic but can also grow in the absence of oxygen, though less abundantly; most bacteria of medical importance are facultative anaerobes

ii) **Anaerobic bacteria**, such as clostridia, grow in the absence of oxygen; **obligate anaerobes** cannot survive in oxygen

iii) **Microaerophilic** bacteria are those that grow best in the presence of **low oxygen tension**, e.g., *Helicobacter*

The reason for the apparent toxicity of oxygen for anaerobic bacteria is not well understood.

One possible reason for this could be the accumulation of hydrogen peroxide along with other toxic peroxides. Most aerobes produce the enzyme catalase which breaks down hydrogen peroxide, whereas anaerobes do not produce this enzyme.

Another reason could be that obligate anaerobes possess essential enzymes that are active only in the reduced state.

Aerobic bacteria obtain their energy and intermediates only through oxidation with oxygen as the ultimate hydrogen acceptor. In aerobic respiration, the carbon and energy source may be completely oxidised to carbon dioxide and water.

Anaerobes use hydrogen acceptors, nitrates or sulphates instead of oxygen (anaerobic respiration). Facultative anaerobes may act in both ways. In these organisms, energy is provided by the production of energy-rich phosphate bonds and the conversion of adenosine diphosphate (ADP) to adenosine triphosphate (ATP). This process is known as **oxidative phosphorylation**.

Fermentation is a process of anaerobic metabolism that leads to the formation of organic end products such as acids, alcohols and gases (carbon dioxide and hydrogen). During the process of fermentation, energy-rich phosphate bonds are produced by incorporating organic phosphates into intermediate metabolites. This process is known as **substrate-level phosphorylation**. The phosphates are used to convert ADP to ATP.

Classification based on temperature requirements

Bacteria vary in their requirement of temperature for growth and have a distinct ‘temperature range’. **Optimum temperature is** the temperature at which growth occurs best.

- **Pathogenic bacteria** have an optimum temperature of 37°C.
- **Mesophilic bacteria** grow best at temperatures of 25–40°C. All bacteria are mesophilic. Within the group of mesophilic bacteria, some like *Pseudomonas aeruginosa* have a wider range (5–43°C), while others like the gonococcus have a restricted range (30–39°C).
- **Psychrophilic** bacteria grow best at temperatures below 20°C, some as low as –7°C. They are soil and water saprophytes and,

though not of direct medical importance, may cause spoilage of refrigerated food, e.g., *Chryseobacterium* and *Pseudomonas*.

- **Thermophiles** grow best at high temperatures of 55–80°C. They may cause spoilage of underprocessed canned food. Some thermophiles (like *Bacillus stearothermophilus*) form spores that are exceptionally thermoresistant. Extremely thermophilic bacteria can grow at temperatures as high as 250°C.

Thermal death point is the lowest temperature that kills a bacterium under standard conditions in a given time. Under moist conditions, most vegetative, mesophilic bacteria have a thermal death point between 50 and 65°C and most spores between 100 and 120°C.

At low temperatures, some species die rapidly though most survive well. Storage in the refrigerator (3–5°C) or the deep freeze cabinet (–30 to –70°C) preserves cultures for a variable length of time. **Rapid freezing** as with solid carbon dioxide or the use of a stabiliser such as glycerol minimises the death of cells on freezing.

Other Factors Affecting Bacterial Growth

Moisture and drying

Water is an essential ingredient of bacterial protoplasm and hence drying is lethal to cells. However, the effect of drying varies in different species. Some delicate bacteria like *Treponema pallidum* are highly sensitive, while others like staphylococci withstand drying for months. Spores are particularly resistant to desiccation and may survive in the dry state for several decades.

Lyophilisation/Freeze-drying/Cryodesiccation is a dehydration process typically used to preserve bacteria, virus and other biological material. This is done by drying the organisms in a vacuum at low temperatures.

Hydrogen ion concentration

Bacteria are sensitive to variations in pH. The majority of pathogenic bacteria grow best at neutral or slightly alkaline conditions (pH 7.2–7.6). Some acidophilic bacteria such as lactobacilli grow under acidic conditions. Strong solutions of acid or alkali (5% hydrochloric acid or sodium hydroxide) readily kill most bacteria, though mycobacteria are

exceptionally resistant to them.

Light

Bacteria (except the phototrophic species) grow well in the dark. They are sensitive to ultraviolet light and other radiation. Photochromogenic mycobacteria form a pigment only on exposure to light and not when incubated in the dark.

Osmotic effect

Bacteria are more tolerant to osmotic variation than most other cells due to the mechanical strength of their cell walls.

Mechanical and sonic stress

Though bacteria have tough cell walls, they may be ruptured by mechanical stress and exposure to ultrasonic vibration.

BACTERIOCINS

Gratia (1925) observed the production of a highly specific bacterial substance by one strain of *E. coli* which was active against another strain of the same species. As it was produced by *E. coli*, the substance was called colicin. Other members of the family Enterobacteriaceae and other bacteria were also found to produce similar substances. The generic name **bacteriocin** was proposed for the highly specific antibiotic-like substances produced by certain strains of bacteria which are active against other strains of the same or different species, e.g., **colicins** from *E. coli*, **pyocins** from *Ps. pyocyanea* (*aeruginosa*), **megacins** from *B. megaterium* and **diphthericins** from *C. diphtheriae*.

Bacteriocins are generally proteins but some may have associated lipopolysaccharide-derived cell walls. Bacteriocins and bacteriophages (viruses that infect bacteria) resemble each other in a number of respects as shown in Table 2.6.

Table 2.6 Characteristics of bacteriocins and bacteriophages

Characteristics	Bacteriocins	Bacteriophages
Nature	Bacterial products	Virus
Attach to specific cell-surface receptors	Yes	Yes

Electron microscopy	Appear like the tail structures of phages	Complete viral structure
Typing of bacteria	Bacteriocin typing	Phage typing
Bacteria that can be typed	*S. sonnei, Proteus* spp. and *P. aeruginosa*	*Staphylococcus, Salmonella*

Colicinogenic Factors

The **synthesis** of bacteriocins in bacteria is determined by the presence of colicinogenic factors (**Col factors**). Col factors are episomes and can be transmitted from cell to cell by conjugation or transduction. Certain physical and chemical agents (UV rays and nitrogen mustard) induce colicin production by cells harbouring Col factors.

A cell producing a bacteriocin is immune to it but may be sensitive to bacteriocins of other closely related strains. Bacteriocins have very specific activity on bacteria, being capable of killing some but not all strains of a species.

Bacteriocin Typing

This is based on the ability of the bacteriocins produced by the test strain to kill standard indicator strains of bacteria. The test bacterium is inoculated as a broad streak on the centre of a culture medium, the bacterial growth is scraped off and the remaining cells killed by exposure to chloroform vapour. Standard indicator strains of bacteria are then streaked at right angles to the original inoculum. After incubation, the pattern of inhibition of the indicator strains represents the bacteriocin type of the test bacterium. However, this method is not routinely used.

Key points

- Bacteria and blue-green algae are prokaryotes; fungi, other algae and protozoa are eukaryotes.
- Bacteria possess a single, circular chromosome.
- The morphological study of bacteria requires the use of microscopes.
- The microscopes currently in use are:
    - Optical or light microscope (bright-field microscope)
    - Phase-contrast microscope
    - Fluorescence microscope

    - Dark-field/dark-ground microscope
    - Electron microscope
- Staining techniques used for the study of bacteria are:
    - Simple stains (methylene blue, basic fuchsin), where all bacteria are stained the same colour
    - Negative stains
    - Impregnation methods
    - Differential stains, where stains impart different colours to different bacteria or bacterial structures (Gram stain, acid-fast stain)
- Based on their shape, bacteria are classified into:
    - Cocci (spherical or oval)
    - Bacilli (rod-shaped)
    - Vibrios (comma-shaped curved rods)
    - Spirilla (rigid spiral forms)
    - Spirochetes (flexuous spiral forms)
    - Actinomycetes (branching filamentous bacteria)
- A bacterial cell has a:
    - Rigid cell wall
    - A phospholipid cytoplasmic membrane
    - Cytoplasm containing nucleoid, ribosomes, mesosomes
    - Flagella
    - Fimbriae and pili
- The gram-positive cell wall is 90% peptidoglycan while the gram-negative cell wall is composed of lipopolysaccharide, which also contains lipid A, a toxic substance that imparts the pathogenic virulence associated with some gram-negative bacteria.
- Bacteria divide by binary fission. The time interval between two cell divisions is called the generation or population doubling time. This may vary from 20 minutes (coliform bacilli) to 20 hours (tubercle bacilli) to 20 days (lepra bacilli).
- The bacterial growth curve consists of a:
    - Lag phase (no appreciable increase in number)
    - Log phase (an exponential increase in bacterial number)
    - Stationary phase (no increase or decrease in number)
    - Decline phase (decrease in the bacterial population due to cell death)
- Bacteria vary in the temperature they require for growth. Mesophilic bacteria grow best at temperatures of 25–40°C, psychrophilic bacteria at temperatures below 20°C and thermophilic bacteria at high temperatures of 55–80°C.

Questions

Short Answers

1. Principles of Gram staining
2. Labelled diagram of the structure of a bacterial cell
3. L-forms of bacteria and their clinical relevance

4. Bacterial cell wall (gram-positive, gram-negative)
5. Structure of flagella
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CHAPTER 3

Sterilisation and Disinfection

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- Physical agents
- Chemical agents
- Gaseous agents
- Applications in the hospital

INTRODUCTION

Microorganisms are ubiquitous in nature. They are found in the surroundings, on inanimate objects and on the surface of the human body. Since they are responsible for infection, contamination and decay, it is necessary to remove or destroy them in health care settings and other human habitats.

Some Definitions

Sterilisation The process by which an article, surface or medium is freed of all living microorganisms in the vegetative or spore state is called sterilisation. They are either eliminated, inactivated or killed. The method of sterilisation used depends on the purpose for which it is carried out, the material to be sterilised and the nature of the microorganisms to be removed or destroyed.

Disinfection The process of destruction of all pathogenic organisms is called disinfection. Some disinfectants may not be able to destroy spores.

Asepsis It is the state of complete absence of viable pathogenic microorganisms in any environment.

Antiseptics Agents that can be safely applied on the skin or mucous membrane to prevent infection by inhibiting the growth of bacteria are called antiseptics.

Bactericidal agents (or germicides) These are substances that can kill bacteria.

Bacteriostatic agents These are substances that prevent the multiplication of bacteria but do not necessarily kill them. With the use of these agents, bateria may remain viable for some period of time. A chemical that is bacteriostatic at a particular concentration may become bactericidal at another concentration.

Decontamination It is the process of rendering an article, area, object or body surface free of contaminants, including microbial, chemical, radioactive and other hazardous materials.

STERILISING AGENTS

Sterilising agents are used to completely eliminate microorganisms and their spores from articles, surfaces or mediums. Several methods are used in health care settings to sterilise instruments, devices and other articles that come into contact with the patients' skin or mucous membranes. Table 3.1 shows some of the common methods of sterilisation in a health care setting.

Some of the agents mentioned in the table are also used as disinfectants.

Table 3.1 Common methods of sterilisation in a health care setting

Physical methods	**Process**
Dry heat	Flaming, incineration or using hot air
Moist heat	Boiling, steam at atmospheric pressure, steam above atmospheric pressure
Filtration	Candles, membranes
Radiation	Ionising and non-ionising radiations
Chemical methods	**Agents**
Alcohols	Ethyl, isopropyl, trichlorobutanol
Aldehydes	Formaldehyde, glutaraldehyde
Ortho-phthalaldehyde	OPA
Peracetic acid	5–15% peracetic acid
Hydrogen peroxide	Chlorox
Hypochlorous acid	Na hypochlorite

Dyes	Aniline dyes and acridine dyes
Halogens	Chlorine and iodine
Phenols	Lysol and cresol
Surface-active agents	Triton, Tween
Metallic salts	Mercuric chloride
Gases	Ethylene oxide, formaldehyde, beta-propiolactone

A. PHYSICAL AGENTS

Heat

The most reliable method of sterilisation is applying heat to an object. Materials that may be damaged at high temperatures can be sterilised by exposing them to low heat for longer periods or by repeated cycles.

Heat can be used for sterilisation in two forms:

1. **Dry heat** kills the microorganism by causing the following changes:

- Protein denaturation
- Damage to the cell by oxidising the molecules
- Destroying cell constituents
- Elevation in the levels of electrolytes, which has a toxic effect

2. **Moist heat** destroys cells by causing the following changes:

- Moist heat results in the denaturation and coagulation of proteins. The advantage of steam is the latent heat that is liberated. When this latent heat condenses on a cooler surface, it raises the temperature of that surface, thereby denaturing its components.
- In the case of spores, steam condenses on them, increasing their water content and resulting in the ultimate hydrolysis and breakdown of the bacterial protein, in addition to killing by high temperature, which is detrimental to the spore.

In a completely moisture-free atmosphere (as with dry heat), bacteria, like many proteins, are more resistant to destruction by heat. They are killed

when the oxidation of the cell constituents occurs at much higher temperatures than that needed for the coagulation of proteins by moist heat.

Thermal death time The time required for sterilisation is inversely proportional to the temperature of exposure and can be expressed as thermal death time. This is the minimum time required to kill a suspension of organisms at a predetermined temperature in a specified environment. It does not include the time taken to reach the specified temperature.

Factors influencing sterilisation

The **factors influencing sterilisation** by heat are:

- Nature of heat—dry or moist
- Temperature and time
- Number of microorganisms present
- Characteristics of the organisms, such as species, strain and the presence of spores
- Type of material from which the organisms must be eradicated
- The presence of organic substances, proteins, nucleic acids, starch, gelatin, sugar, fats and oils, which increases the thermal death time
- The presence of disinfectants and high acid or alkaline pH, which hasten bacterial killing

Dry Heat Sterilisation

There are three methods in use in dry heat sterilisation: flaming, hot air oven and incineration.

Flaming

An inoculating loop or wire, the tips of forceps and searing spatulas can be sterilised by holding them over a Bunsen flame till they become red-hot. Inoculation loops carrying infective material may be dipped in a disinfectant before flaming to prevent spattering.

Hot air oven

This is the most widely used method of sterilisation by dry heat. Sterilisation is achieved by conduction. Heat is absorbed by the surface of the item to be sterilised, which then penetrates to the centre, until the entire item reaches the desired temperature. The oven is usually heated by electricity. A fan is fitted inside to ensure even distribution of air and elimination of air pockets. The chamber should not be overloaded. Free circulation of air in between the objects should be ensured. Glassware

should be perfectly dry before being placed in the oven. Test tubes and flasks should be wrapped in craft paper. Rubber materials (except silicon rubber) cannot withstand the temperature inside a hot air oven. At 180°C, cotton plugs may get charred (Fig. 3.1a, b).

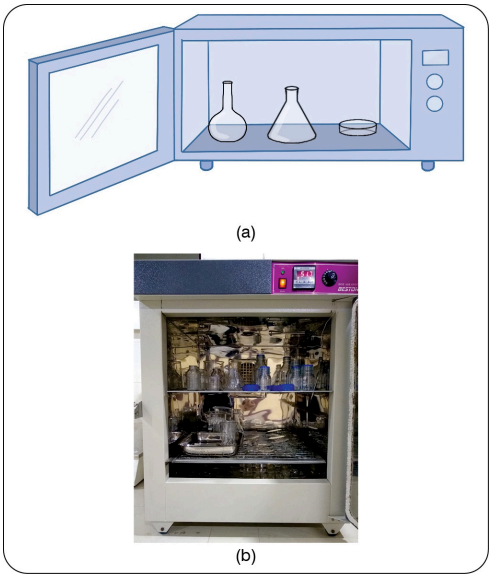


Fig. 3.1 Hot air oven: (a) diagrammatic representation and (b) a photograph (*Source:* Pushpagiri Institute of Medical Sciences and Research Centre, Thiruvalla, Kerala)

The **holding times** for different items are different and are as shown in Table 3.2.

Table 3.2 Holding period of different items

Holding period	Items to be sterilised
Two hours at 160°C	• Glassware, forceps, scissors, scalpels and all glass syringes • Swabs • Pharmaceutical products such as liquid paraffin, dusting powder, fats and grease • Materials made of silicon rubber
Two hours at 150°C	• Sharp instruments, such as those used in ophthalmic surgery
One hour at 150°C	• Oils, glycerol and dusting powder

The oven must be allowed to cool slowly for about two hours before the door is opened since glassware may crack due to sudden or uneven cooling.

Table 3.3 lists the recommended temperature and duration for heat sterilisation.

Table 3.3 Recommended temperature and duration for heat sterilisation

Method	Temperature (°C)	Holding time (in minutes)
Autoclave	121	15
	126	10
	134	3
Hot air oven*	160	120
	170	60
	180	30

*British Pharmacopoeia 1998, European Pharmacopoeia 1990

Incineration

This is an excellent method for terminal sterilisation needed before destroying biomedical waste. Plastics such as PVC and polythene can be dealt with similarly but polystyrene materials emit clouds of dense toxic smoke which pollute the environment.

In order to check the proliferation of poorly designed biomedical waste incinerators, guidelines on '**Design and Construction of Biomedical Waste Incinerator**' recommend various design features of an incinerator as well as air pollution control devices.

Sterilisation control

Physical Temperature monitoring by thermocouples
Chemical A **Browne's tube** (green spot) is used. A green colour produced after 60 minutes at 160°C or 115 minutes at 150°C suggests complete sterilisation.
Biological Heat-resistant spores of a non-toxigenic strain of *Clostridium tetani* or *Bacillus subtilis* subsp. *niger* are used to indicate the efficiency of dry heat sterilisation.

Moist Heat Sterilisation

Moist heat sterilisation can be carried out at temperatures below 100°C, at 100°C and using steam under pressure.

Sterilisation at temperatures below 100°C

Pasteurisation of milk Milk is heated at either 63°C for 30 minutes (the

holder method) or 72°C for 15–20 seconds (the **flash process**), followed by rapid cooling to 13°C or lower. By these methods, all non-sporing pathogens such as mycobacteria, brucellae and salmonellae are destroyed. *Coxiella burnetii* is relatively heat-resistant and may survive the holder method.

Inspissation Media such as Lowenstein–Jensen and Loeffler's serum are rendered sterile by heating them at 80–85°C for half an hour on three successive days in an **inspissator** (Fig. 3.2).

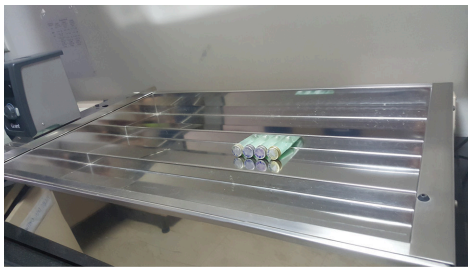


Fig. 3.2 Inspissator (*Source:* Intermediate Reference Laboratory for TB, SCB Medical College, Cuttack)

Vaccine bath Bacterial vaccines are heat-inactivated at 60°C for one hour in special vaccine baths. Serum or body fluids containing coagulable proteins can be sterilised by heating them for one hour at 56°C in a water bath (Fig. 3.3) on several successive days

Fig. 3.3 Shaking water bath (*Source:* Shaking water bath, photograph by Athikhun.suw, July 9, 2015. Distributed under CC BY-SA 4.0 http://creativecommons.org/licenses/by-sa/4.0/)

- All non-sporing bacteria are killed at 60°C in 30 minutes.
- *Streptococcus faecalis* and *Staphylococcus aureus* are killed by heating them at 60°C for 60 minutes.
- Yeasts, moulds and vegetative bacteria are killed at 80°C in 5–10 minutes.
- Spores of *Clostridium botulinum* need to be heated to 120°C for 4 minutes or 100°C for 330 minutes.
- Most viruses are killed rapidly at 60°C except for polio and hepatitis B viruses which may survive at

this temperature for 30 minutes and 10 hours respectively.

Sterilisation at temperature of 100°C

Boiling Vegetative bacteria are killed almost immediately at 90–100°C but bacterial spores can withstand long periods of boiling. The material to be sterilised is immersed in the water and boiled for 10–30 minutes. The lid of the steriliser should not be opened during this period. **Boiling is NOT recommended for sterilisation of instruments used for surgical procedures.** Hard water should not be used for boiling. Addition of 2% sodium bicarbonate to the water may render it soft and make it suitable for sterilisation.

Free steam at atmospheric pressure (100°C) An atmosphere of **free steam** is used to sterilise culture media, which may decompose if subjected to higher temperatures. This can be done in an autoclave, which generates free steam. The valves are left open so that pressure does not build up inside the chamber, thus preventing the temperature from rising above 100°C.

Tyndallisation or intermittent sterilisation This method is used for media containing sugars or gelatin. The media is exposed to a temperature of **100°C for 20 minutes on three successive days**. The principle of this method is that the first exposure kills all vegetative bacteria, while the spores, which germinate subsequently (during the resting period) because of the favourable medium they are in, are killed on subsequent exposure to 100°C for 20 minutes. However, this method may fail to kill spores of certain anaerobes and thermophiles.

Sterilisation using steam under pressure

Autoclave (steam steriliser)

The principle of the autoclave or steam steriliser is that when water boils, its vapour pressure equals that of the surrounding atmosphere. When the pressure inside a closed vessel increases, the temperature at which water boils also increases, generating steam at a higher temperature. When this steam comes into contact with a cooler surface, it condenses and transmits its latent heat to that surface (1,600 mL steam at 100°C and at atmospheric pressure condenses into 1 mL of water at 100°C and releases 518 calories of heat). The large reduction in volume sucks in more steam and the process continues till the temperature of that surface becomes equal to that of steam.

Condensed water ensures moist heat for killing microbes by denaturing the macromolecules, particularly proteins.

Sterilisation by steam under pressure is carried out at temperatures between 108°C and 147°C. Materials such as linen, instruments, laboratory ware, media and pharmaceutical products can be sterilised in an autoclave. Aqueous solutions are sterilised between 108°C and 126°C. Heat is conducted through the walls of the sealed containers until the temperature of the fluid inside is the same as that of the steam outside.

Types of autoclaves Two types of autoclaves are available:

i) **Gravity displacement sterilisers,** where steam enters the **steriliser chamber** and displaces the residual air through an open vent

ii) **High vacuum sterilisers**, where the sterilisation of unwrapped items is done in a **pre-vacuum chamber**

Several types of autoclaves **(steam sterilisers)** are used in health care settings (Table 3.4).

Table 3.4 Autoclaves used in health care settings

Type	Uses
Laboratory autoclave	For sterilisation of laboratory ware and media
Hospital dressing sterilisers	Best suited for parenteral solutions, injectables and hospital dressings
Instrument sterilisers	Used to sterilise surgical and dental instruments
Rapid cooling sterilisers	May be used for hospital garments, parts of equipment, accessories, containers, culture media, laboratory waste and bulk drugs

Laboratory autoclave It consists of a vertical or horizontal cylinder of gunmetal or stainless steel, enclosed in a supporting sheet iron case. The lid is fastened by screw clamps and made airtight. An exit tap for air and steam, and a pressure gauge are attached to the autoclave. A safety valve, which can be set to blow off if pressure rises beyond the desired level, is also attached to the autoclave (Fig. 3.4a and b).

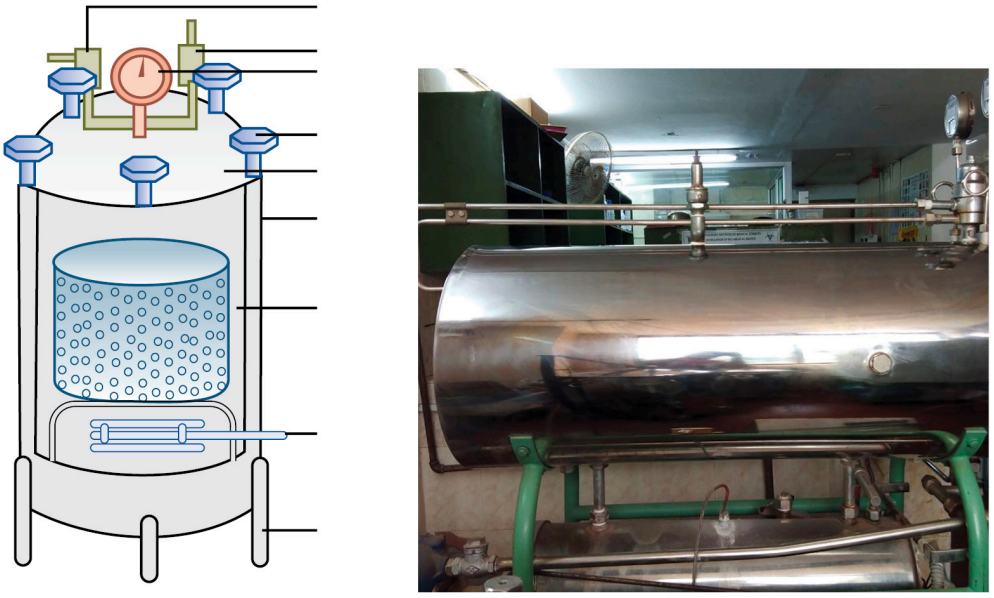


Fig. 3.4 (a) a simple vertical autoclave (1—Release valve; 2—Safety valve; 3—Pressure gauge; 4—Tightening screws; 5—Cover; 6—Body; 7—Bin containing material; 8—Heating element; 9—Stand) and (b) horizontal autoclave (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Mechanism Sufficient water is added to the cylinder, and the material to be sterilised is placed on a tray within a bin. The autoclave lid is screwed tight with the discharge tap open. The autoclave is then heated by electricity. The steam–air mixture is allowed to escape freely till all the air has been displaced (tested by leading the escaping steam into a pail of water through rubber tubing). When all the air has been expelled, the discharge tap is closed. The steam pressure rises inside till the desired set level, the safety valve then opens and the excess steam escapes. From this point, the **holding period** is calculated. When the holding period is over, the heater is turned off and the autoclave is allowed to cool till the pressure reaches atmospheric pressure. Finally, the discharge tap is opened slowly and air is let into the autoclave.

Safety precaution
If the tap is opened when the internal pressure is high, liquid media boils violently, spills from the container and may even explode. If opened after the pressure inside has fallen below atmospheric pressure, an excessive amount of water will evaporate and be lost from the media.

The **drawbacks** of this type of autoclave are:

- The method of **air discharge is inefficient**, and it is difficult to decide when the discharge is complete.
- Materials remain **moist** after removal from the autoclave.

Vacuum sterilisers do not have this disadvantage as they have automatic cycle control. The air is drawn out, whereby steam penetrates faster and the time required for sterilisation is shorter. Once the sterilisation is over, post-cycle vacuum can be programmed for quick drying.

The pressure cooker serves as a miniature autoclave and may be used for sterilising small articles in clinics and small establishments.

Sterilisation control The control of sterilisation is similar to that of dry heat sterilisation (as described above) except for the use of ***Geobacillus stearothermophilus***, which is used as the test organism for checking sterilisation by steam under pressure. This is a thermophilic organism with an optimum growth temperature of 55–60°C; its spores require an exposure of 12 minutes at 121°C to be killed.

Biological indicators are the only process indicators that directly monitor the lethality of a given sterilisation process. Rapid readout biological indicators, which provide results within one to three hours, are available for a shorter turnaround time for reading the results (Table 3.5).

Table 3.5 Biological indicators and their uses

Organism	Use
Geobacillus stearothermophilus	Moist heat
Bacillus subtilis var. *niger*	Dry heat
B. subtilis var. *globigii*	Ethylene oxide
B. pumilus	Ionising radiation
Pseudomonas diminuta	Filtration

Cold Sterilisation

This is a process by which materials are sterilised at low temperatures by:

- Membranes (filters)
- Radiation
- Chemicals (as disinfectants)
- Other means excluding high temperature

Filtration

Filtration helps remove bacteria from heat-labile liquids such as sera and solutions of sugars or antibiotics. As viruses pass through ordinary membrane filters, the materials sterilised by this method are only free of bacteria.

Types of filtration

Membrane filtration

Membrane filters made of cellulose esters or other polymers have largely replaced other types of filters. They are routinely used in water purification and analysis, sterilisation and sterility testing, and for the preparation of solutions for parenteral use. They come in a wide range of **average pore diameters** (APD). The pore size of microfiltration membranes is in the range of 0.1–5 ∝m, the 0.22 ∝m size being the most widely used for filtering out bacteria (Fig. 3.5).

Fig. 3.5 Membrane filter with filter pads (Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Ultrafiltration (UF)

UF membranes have a pore size range of 0.01–0.1 ∝m. UF membranes reject particles such as silica, viruses, endotoxins and proteins.

Nanofiltration (NF)

NF membranes have a pore size range of 0.001–0.01 ∝m. NF membranes can filter salts, sugars and dyes.

High-efficiency particulate air (HEPA) filter

This is a type of **air filter** used for removal of at least 99.97% of airborne particles up to 0.3 ∝m in diameter. Particles that are larger are filtered with high efficiency. These filters are used in biosafety cabinets, operation theatres, etc., to restrict the entry of infectious particles into the air.

An ultra-low penetration air (**ULPA**) filter can (theoretically) remove from the air at least 99.99% of dust, pollen, mould, bacteria and any airborne particles with a size of 120 nanometres or more. These are used in the precision industry.

Sterilisation control

Serratia marcescens and *Brevundimonas diminuta* are two bacteria that are used to test the pore size of a filter. A filter can be deemed effective on the basis of its ability to prevent a bacterial challenge of 1 × 10 CFU (colony forming units) of *Brevundimonas diminuta* per cm^2 of filter surface to pass through. Generally, the pore size that prevents the bacteria from passing through is estimated to be 0.2 to 0. 22 ∝m.

Radiation

Two types of radiation are used for sterilisation:

Non-ionising radiation

These are electromagnetic rays with wavelengths longer than those of visible light. These rays produce **low-energy radiation**. These are, to a large extent, absorbed as heat.

Infrared radiation is used for rapid mass-sterilisation of pre-packed items such as disposable plastic syringes and catheters.

Ultraviolet radiation is used for disinfecting enclosed areas such as biosafety cabinets in laboratories, entryways, operation theatres and laboratories.

Ionising radiation

These are high-energy radiations like X-rays, gamma rays and cosmic rays, which are highly lethal to DNA and other vital constituents. They have a very high penetrative power. Since there is no appreciable increase in temperature in this method, it is also referred to as cold sterilisation.

Gamma radiation is used by commercial plants for sterilising items like plastics, syringes, swabs, catheters, animal feeds, cardboard, oils, greases, fabric and metal foils.

B. CHEMICAL AGENTS

An ideal antiseptic or disinfectant

An ideal antiseptic or disinfectant should:
- Have a wide spectrum of activity and be effective against all microorganisms
- Be active in the presence of organic matter
- Be effective in acid as well as alkaline media
- Have speedy action
- Have high penetrating power
- Be stable

Factors that afffect the potency of a disinfectant

- Concentration of the substance
- Contact period
- pH of the medium
- Temperature
- Nature of the organisms
- Presence of extraneous material

Mode of action of chemical agents

- By protein coagulation
- By disruption of the cell membrane, which results in exposure, damage or loss of contents
- By removal of free sulphydryl groups essential for the functioning of the enzymes
- By substrate competition for enzymes necessary for the metabolism of the cell

Several chemical agents are used as antiseptics and disinfectants.

1. Alcohols

- ***Ethyl alcohol (ethanol) and isopropyl alcohol*** These are the most frequently used. They are used mainly as skin antiseptics at a concentration of 60–90% in water. They act by denaturing bacterial proteins and have no effect on spores. Protein slows the action of this agent, whereas 1% mineral acid or alkali enhances it. Isopropyl alcohol is preferred over ethyl alcohol as it is a better

fat solvent and is more bactericidal and less volatile. It is used for the disinfection of clinical thermometers.

- ***Methyl alcohol*** This is effective against fungal spores and is used for cleaning cabinets and incubators. To do this, a pad moistened with methanol and a dish of water (to ensure high humidity) are kept inside the chamber, which is left at working temperature for several hours. Methyl alcohol vapour is toxic and inflammable.

2. Aldehydes

- ***Formaldehyde*** This is active against the amino group in the protein molecule. In aqueous solutions, it is markedly bactericidal, sporicidal and virucidal.

Uses of formaldehyde gas

- It preserves anatomical specimens.
- It destroys anthrax spores in hair and wool.
- 10% formalin containing 0.5% sodium tetraborate is used to sterilise instruments, heat-sensitive catheters, and for fumigating wards, isolation rooms and laboratories.
- Under properly controlled conditions, clothing, bedding, furniture and books can be satisfactorily disinfected.

Formaldehyde gas is generated by adding 150 g of potassium permanganate to 280 mL formalin for every 1,000 cu. ft (28.3 cu. m) of room volume. The sealed room is left unopened for 48 hours after fumigation. Then, the gas is neutralised with ammonia (300 mL for every litre of formaldehyde used). Fumigation of operation theatres is no longer preferred.

- ***Glutaraldehyde*** This is a disinfectant that has a mode of action that is similar to that of formaldehyde. It is especially effective against tubercle bacilli, fungi and viruses. It has no deleterious effect on the cement or the lenses of instruments.

Uses of glutaraldehyde gas

It is used to sterilise the following items:
- Cystoscopes
- Bronchoscopes
- Rubber anaesthetic tubes
- Plastic endotracheal tubes
- Polythene tubing
- Metal instruments

- ***Ortho-phthalaldehyde*** It has bactericidal activity. It is used to cleanse endoscopes between patients as it is quick, effective and safe.
- ***Peracetic acid*** This chemical agent has a good bactericidal activity, particularly against common antibiotic-resistant bacteria such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus* and *Clostridium difficile*.
- ***Hypochlorous acid*** This acid is generated from the reverse reaction of sodium hypochlorite and hydrogen peroxide. It has bactericidal activity against common pathogenic organisms. It is active against biofilms and microorganisms within the biofilms.

3. Dyes

Two groups of dyes, aniline and acridine, are used extensively as skin and wound antiseptics. Both are bacteriostatic in high dilution but have low bactericidal activity. They are more active against gram-positive than gram-negative bacteria.

- ***Aniline dyes*** These include brilliant green, malachite green and crystal violet. They do not act against tubercle bacilli. Hence malachite green is used in the Lowenstein–Jensen medium as a selective agent. They are non-irritant and non-toxic to the tissues. They are inhibited by organic material. Their lethal effects on bacteria are believed to be due to their reaction with the acid groups in the cell.

- ***Acridine dyes*** These are not as selective as the aniline dyes. They are minimally affected by the presence of organic matter. Important dyes in this group are proflavine, acriflavine, euflavine and aminacrine. They impair the DNA complexes of the organisms and prevent replication.

4. Halogens

- ***Iodine*** Iodine in an aqueous and alcoholic solution has been widely used as a skin disinfectant (e.g., tincture iodine). It is bactericidal, with moderate action against spores. It is active against tubercle bacteria and viruses. Compounds of iodine with non-ionic wetting or surface-active agents known as **iodophores** have better action than aqueous or alcoholic solutions of iodine.
- ***Chlorine*** Chlorine and its compound hypochlorite have been used as disinfectants over time. They are markedly bactericidal and virucidal. Town water supplies, swimming pools and food and dairy industries use chlorine for disinfection. Organic chloramines are used as antiseptics for dressing wounds.

Disadvantages of chlorine

Certain types of microorganisms have shown resistance to low doses of chlorine. Some parasites like giardia may not get eliminated with chlorine. High doses of chlorine may cause diarrhea and other GI disturbances. Chlorine is also a skin irritant.

5. Phenols

These compounds are obtained by the distillation of coal tar at temperatures between 170°C and 270°C. The bactericidal effect of phenols is due to their ability to damage the cell membrane and inactivate membrane-bound oxidases and dehydrogenases, thereby causing the lysis and death of the microorganism. Low concentrations of phenol precipitate proteins.

- ***Phenol (carbolic acid)*** This is widely used as a disinfectant in hospitals. The commonly used compounds are **Lysol** and **cresol,** which are active against a wide range of organisms. They are not readily inactivated by the presence of organic matter; hence they are good general disinfectants. Various proprietary preparations or formulations of phenol are in wide use.
- ***Chlorophenols and chloroxyphenols*** While these are less toxic and irritant, they are also less active (inactive against *Pseudomonas*) and readily inactivated by organic matter. They are active against most gram-positive organisms and fairly effective against gram-negative bacteria. Chlorhexidine (hibitane) is a relatively non-toxic skin antiseptic. Hexachlorophene, on the other hand, is potentially toxic and should be used with care.

6. Gases

- ***Ethylene oxide*** This is a colourless liquid with a boiling point of 10.7°C and good penetrating ability at normal temperature and pressure. It has a sweet, ethereal smell, and is highly inflammable and explosive at concentrations greater than 3%. Combination with 10% carbon dioxide or nitrogen makes it less explosive.

It acts by alkylating the amino, carboxyl, hydroxyl and sulphydryl groups of protein molecules within the microbes and spores. It also reacts with DNA and RNA (which makes it virucidal). Its mutagenic and carcinogenic property makes it toxic to humans. A gas steriliser using ethylene oxide is shown in Fig. 3.6.

Fig. 3.6 Ethylene oxide gas steriliser (Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Items sterilised using ethylene oxide

Ethylene oxide diffuses through several types of porous materials and readily penetrates some plastics. It is especially used for sterilising heat-sensitive equipment such as:

- Heart–lung machines
- Respirators
- Suture materials
- Dental equipment
- Books and clothing

Ethylene oxide has a wide range of applications within and outside the hospital. Since it is an irritant, personnel working with it have to take strict precautions.

- ***Beta-propiolactone (BPL)*** This is an organic compound of the lactone family and is made up of four carbon rings. It reacts with water to produce hydracrylic acid. It is no longer used for fumigation as it is carcinogenic.
- ***Hydrogen peroxide fogging*** This method has replaced fumigation. It acts as a bactericide by oxidising the cell wall of the organism. It has the advantage of short cycle time and is non-toxic.

7. Surface-Active Agents

Surface-active agents are substances that alter the energy relationship at interfaces, producing a reduction in surface tension (Table 3.6). They are widely used as wetting agents, detergents and emulsifiers.

Mechanism These substances act on the phosphate groups of the cell membrane and also enter the cell. As a result, the membrane loses its semi-permeability and the cell proteins are denatured. They act on bacteria but have no action on spores, tubercle bacilli and most viruses.

The common surface-active compounds are **acetyl trimethyl ammonium bromide** (cetavlon or cetrimide) and **benzalkonium chloride**. They are most active at alkaline pH. Organic matter reduces their action and anionic surface-active agents, like ordinary soaps, render them inactive. Anionic surface-active compounds, like common soap, have moderate action.

Soaps prepared from saturated fatty acids (such as coconut oil) are more effective against gram-negative bacilli while those prepared from unsaturated fatty acids (oleic acid) have greater action against gram-positive and the *Neisseria* group of organisms.

Table 3.6 Classification of surface-active agents

Category	Agents
Anionic	Sulphide, fluoride, bromide, iodide
Cationic	Sodium, iron, lead
Non-ionic	Polyoxyethylene (e.g., Tween, Triton)
Amphoteric	Tego compounds

8. Metallic Salts

Though all salts have a certain amount of germicidal action, salts of heavy metals are quite effective. Silver, copper and mercury salts are used as disinfectants. They are protein coagulants and have the capacity to combine with the free sulphydryl groups of cell enzymes. **Thiomersal**, **phenyl mercury nitrate** and **mercurochrome** are less toxic and hence used as mild antiseptics. They have marked bacteriostatic, weak bactericidal and limited fungicidal action. Copper salts are used as fungicides.

The commonly used methods of sterilisation are presented in Table 3.7 and the methods of antisepsis/disinfection are presented in Table 3.8.

Table 3.7 Commonly used methods of sterilisation

Method	Types/Equipment	Agents for which used
Dry heat	Flaming	Inoculating loop/wire, tips of forceps, searing spatulas
	Hot air oven	Glassware, all glass syringes, sharp instruments, liquid paraffin, dusting powder, fats and grease
Moist heat	Below 100°C: • Pasteurisation • Inspissation • Vaccine baths	Milk Lowenstein–Jensen medium, Loeffler's serum slope Vaccines and sera
	100°C: • Boiling • Tyndallisation or intermittent sterilisation at 100°C for three consecutive days	Material used for domestic appliances, baby bottle, teats and caps Media containing sugars or gelatin
	Steam at atmospheric pressure: Steam sterilisers (Koch or Arnold steamer)	Culture media that are sensitive to higher temperature
	Steam under pressure: Autoclave • Gravity displacement type • High vacuum type	Hospital linen, instruments, laboratory ware, media and pharmaceutical products
Filtration	Sintered glass filters Asbestos filters (Seitz—not recommended due to carcinogenic property) Candle filters Membrane filters	Water purification and analysis, sterilisation and sterility testing of liquids Solutions for parenteral use
Cold sterilisation	Radiation: Ionising • X-rays • Gamma rays • Cosmic rays	Plastics, syringes, swabs, catheters, oils, grease Industrial use: animal feeds, fabric and metal foils
	Non-ionising • Infrared	Mass pre-packed materials: plastic syringes, catheters

	• Ultraviolet	Laboratory cabinets, closed chambers
Liquid chemical sterilising agents	Glutaraldehyde Ortho-phthalaldehyde Peracetic acid Hydrogen peroxide Hypochlorous acid	Cystoscopes, endoscopes, tubings and metal instruments

Table 3.8 Methods of antisepsis/disinfection (by chemical agents)

Agents	Types	Use
Alcohols	Ethyl alcohol (ethanol) Isopropyl alcohol Methyl alcohol	Skin antiseptics Cleaning cabinets and incubators
Aldehydes	Glutaraldehyde	Sterilising instruments, heat-sensitive catheters, cystoscopes, bronchoscopes, rubber anaesthetic tubes, plastic endotracheal tubes and polythene tubing
	Formaldehyde (gas)	Fumigating OTs, wards, isolation rooms and laboratories (not preferred now); under properly controlled conditions, clothing, bedding, etc., can also be sterilised
Dyes	Aniline dyes • Brilliant green • Malachite green • Crystal violet • Acridine dyes • Acriflavine • Proflavine	Used on skin and in some culture media (Lowenstein–Jensen medium) Used as antiseptics on skin
Halogens	Iodine Chlorine Hypochlorites Chlorhexidine (Hibitane)	Skin disinfectant Water supply, swimming pools Disinfectant widely used in hospitals at different concentrations
	Hexachlorophene	Skin antiseptic, wound dressing
Phenols	Lysol and cresol	Work benches, floors and walls
Gas	Ethylene oxide	Heart–lung machines, respirators, suture materials, dental equipment, clothing
	Hydrogen peroxide	Used for fogging

Testing of Disinfectants

There is no single reliable test available to determine the efficiency of a

disinfectant, as several parameters influence its activity. **Traditionally, phenol was taken as the standard, and action of the test agent was compared to it.**

Rideal–Walker test
This test is now of historical importance. It is the ratio of the dilution of any disinfectant that kills a microorganism to the dilution of phenol that kills the microorganism in the same time under identical conditions. It is expressed as **phenol coefficient** (phenol = 1) of the disinfectant. However, this test does not take into account the presence of organic matter. To overcome this disadvantage, the test has been modified into the **Chick–Martin test**, the disinfectant of which acts in the presence of organic matter (dried yeast or feces).

Various other modifications have been introduced, but no test is entirely satisfactory. For practical purposes, the **Kelsey–Sykes** or **in-use** test is performed to assess the effectiveness of disinfectants. In these tests, working solutions of the disinfectants used in the hospital are sampled at different times during their use. Table 3.9 lists the concentrations used for disinfectants in clinical practice.

Table 3.9 Commonly used concentration of disinfectants

Name of the disinfectant	Concentration
Glutaraldehyde	2%
Phenol	5%
Sodium hypochlorite	0.5–5%*
Chlorhexidine	1–4%*
Povidone-iodine	10%
Alcohol	70–80%

* Concentration will vary with the clinical situation

STERILISATION AND DISINFECTION IN A HEALTH CARE SETTING

Sterilisation and disinfection form key components of ensuring patient safety in a health care setting. Spaulding's classification has been used to classify the devices used on the patient and determines the type of method to be used for their disinfection or sterilisation.

Spaulding's Classification

Critical items

Critical items are those which enter sterile tissue or the vascular system and pose a high risk of infection if the article is contaminated. This category includes surgical instruments, cardiac catheters, implants, etc. They must be sterilised by autoclaving if heat-stable or with ethylene oxide or hydrogen peroxide gas plasma if heat-sensitive.

Semi-critical items

Articles that come in contact with mucous membranes or non-intact skin are called semi-critical items. They require high-level disinfection. Glutaraldehyde is the most commonly used high-level disinfectant. Endoscopes and bronchoscopes undergo high-level disinfection prior to and between patients. Some semi-critical items like thermometers and blood pressure cuffs for neonates require only intermediate-level disinfection. This is done by disinfecting the instruments with alcohol since the articles may not be compatible with glutaraldehyde.

Non-critical items

These items come in contact with intact skin but not the mucous membranes. Examples are bedpans, blood pressure cuffs, bed rails, bedside tables, etc. They can be cleaned or treated with low-level disinfectants since they carry no risk of transmitting microorganisms to the patients directly.

High-level disinfectant This is a chemical that kills all microbial pathogens except large numbers of spores. It may have some activity against a smaller number of spores if the contact time is increased, e.g., glutaraldehyde and hydrogen peroxide.
Intermediate-level disinfectant This is a chemical that kills all microbial pathogens including mycobacteria and non-enveloped viruses except spores, e.g., alcohol, phenolic compounds and iodophores.
Low-level disinfectant This is a chemical that kills only vegetative bacteria, fungi and lipid-enveloped viruses, e.g., quaternary ammonium compound (Table 3.8).

New Methods of Sterilisation of Heat-Sensitive Articles

Plasma sterilisation

Plasma is known as the fourth state of matter and consists of ions, electrons or neutral particles. In this method of sterilisation, radio frequency energy is

applied to a gas, creating an electromagnetic field. Into this, hydrogen peroxide vapours are introduced, which generates a state of plasma containing free radicals of hydrogen and oxygen. This state renders the articles in the environment of the plasma sterile by denaturing all microorganisms. Arthroscopes, urethroscopes, etc., are sterilised by plasma sterilisation.

The **Central Sterile Services Department** (**CSSD**) plays an important role in the infection control process of all hospitals and health care settings. This facility is also called sterile processing department (SPD) or central supply department (CSD). This is an integrated place in hospitals and other health care facilities, where physical cleaning, washing, packing and sterilisation of all hospital linen, medical devices, surgical instruments and equipment are carried out.

There is a unidirectional work flow with unsterile and soiled linen and instruments received at one end, followed by physical cleaning, washing and drying. This is followed by packing and sterilisation. The equipment used in this process are autoclaves, ethylene oxide (ETO) and plasma sterilisers.

The packed materials are marked with a chemical indicator on the surface which indicates the status of sterilisation. Along with the date and time, the validity of the packed material is also indicated.

A workflow diagram of a hospital CSSD

Unsterile material → Physical cleaning → Washing → Drying → Packing → Sterilisation (autoclave/ETO/plasma sterilisation) → Checking for sterility → Distribution to end users (OTs, wards, etc.)

Key points

- **Sterilisation** is the process by which an article, surface or medium is freed of all living microorganisms, either in the vegetative or spore state.
- **Disinfection** is the destruction or removal of all pathogenic organisms, or organisms capable of giving rise to infection.
- The factors that determine the type of sterilising or disinfecting process to be used include:
    - Time
    - Temperature
    - Stage of growth of the organism
    - Nature of the medium in which the organism is present and number of organisms present

- **Physical methods** of sterilisation use:
    - Heat (dry heat, moist heat)
    - Filtration
    - Radiation
- **Chemical methods** include alcohol, aldehyde, dyes, halogens, phenol, surface-active agents and gases.
- **Spaulding's classification** categorises devices used on the patient as:
    - Critical, semi-critical and non-critical items

Questions

Essays

1. Describe the principle and functioning of an autoclave. Mention the uses of an autoclave in a health care setting.
2. Enumerate the agents used for disinfection in hospitals. Describe their mechanism of action.

Short Answers

1. Process and use of pasteurisation
2. Agents and use of antiseptics
3. Uses of gamma radiation for sterilisation
4. Indicators for sterilisation
5. Principle of plasma sterilisation
6. Methods of cold sterilisation

CHAPTER 4

Culture and Identification of Bacteria

Chapter navigation

- Media for bacterial cultivation
- Growth of bacteria on culture media
- Staining of bacteria
- Identification based on:
  - Biochemical tests
  - In vivo pathogenicity tests
  - Immunological tests

INTRODUCTION

Microorganisms exist as a mixed population in clinical material or as resident flora. They need to be cultivated in pure form for several reasons as listed below.

Reasons to cultivate microorganisms in pure form

- To demonstrate their properties so as to identify them for their clinical significance
- To determine their sensitivity to antibiotics
- To study their physiological virulence and genetic properties
- To obtain sufficient growth for preparing antigens and vaccines
- To type isolates for epidemiological purposes
- To archive them for research purposes

To identify clinically important bacteria, the organisms need to be isolated from the samples submitted to the laboratory. This is done by inoculating the samples on growth media required for the bacteria to replicate and form either colonies on solid media or suspensions in liquid media. **This forms**

the starting point in the identification of bacteria and the testing of their susceptibility to antibiotics.

CULTURE MEDIA

Essential Ingredients of Media

- **Peptone is a common ingredient** of culture media. It is a complex mixture of partially digested proteins. Its constituents are **proteoses, polypeptides** and **amino acids**.
- Culture media contains a variety of **inorganic salts** including phosphates, potassium and magnesium.
- Certain accessory **growth factors** such as riboflavin are also present.
- **Blood, serum** and **yeast extract** are other common ingredients of culture media.

Classification

Bacterial culture media are classified (Table 4.1) based on:

A. Their consistency
B. Their constituents
C. The functional requirement by bacteria

Table 4.1 Classification of culture media

S.No.	Type of culture media	Examples
Based on consistency		
1.	Liquid	Brain–heart infusion broth, peptone water and nutrient broth
2.	Solid	Nutrient agar, blood agar and chocolate agar
3.	Simple	Non-nutrient agar and nutrient agar
4.	Complex	Thiosulphate citrate bile salt sucrose agar (TCBS)
5.	Synthetic or defined	Hank's balanced salt solution (used for virus transport)
Based on their functional requirement		

1.	Enriched	Todd–Hewitt broth (for fastidious, nutritionally demanding organisms)
2.	Enrichment	Selenite F medium (for fecal samples to suppress gut commensals)
3.	Selective	Salmonella–Shigella agar (isolates *Salmonella* and *Shigella* in fecal samples)
4.	Indicator	MacConkey agar (differentiates lactose fermenters from non-lactose fermenters)
5.	Differential	Mannitol salt agar (differentiates *Staphylococcus* aureus from *Staphylococcus epidermidis*)
6.	Transport	Stuart's transport medium (for transporting throat swabs and genital tract swabs from which pathogens are to be isolated)
7.	Anaerobic media	Robertson's cooked meat medium and thioglycolate medium (for cultivation of *Bacteroides*, clostridia, etc.)

Classification based on consistency

1. Liquid media

Liquids are used when large volumes of the clinical material need to be inoculated to obtain a culture (e.g., blood). They are also used for preparing bulk cultures of antigens or vaccines. Bacteria grow diffusely in liquids**.**

2. Solid media

On solid media, bacteria have distinct colony morphology and exhibit several characteristic features such as pigmentation (on nutrient agar) or hemolysis (on blood agar), making identification easy.

- **Agar** (or agar-agar) forms the base of solid media. Agar is obtained from a type of seaweed. It has virtually no nutritive value and is not affected by the growth of bacteria. It **melts at 98°C** and usually **solidifies at 42°C** depending on the agar concentration. Approximately 2% agar is used for solid media.

3. Semisolid media (sloppy agar)

This has low concentration of agar (0.5–1%). It may be used to demonstrate motility of the bacteria—motile bacteria exhibit diffuse growth.

Classification based on the constituents

1. Simple media (basal media)

- **Nutrient broth** is an example of simple media. It consists of peptone, meat extract, sodium chloride and water. It is used to demonstrate motility in a bacterial growth.
- **Nutrient agar**, made by adding 2% agar to nutrient broth, is the simplest and most common solid medium used as basal media in clinical, diagnostic laboratories. It is also used for demonstrating the pigment-producing ability of bacteria.

2. Complex media

These have added ingredients for special purposes such as bringing out certain characteristics or for providing special nutrients required for the growth of the bacterium under study.

3. Synthetic or defined media

These media are prepared from pure chemical substances and the exact composition of the medium is fully documented. They are used for various special studies such as metabolic requirements.

Classification based on functional requirements

1. Enriched media

Nutritional requirements, such as blood, serum or eggs are added to a basal medium to cultivate exacting or fastidious bacteria. Examples are blood agar, chocolate agar and brain–heart infusion broth (BHIB).

Blood agar This is a solid culture medium consisting of agar, peptone and blood. Though sheep blood is usually used, horse, cow and pig blood may also be used.

Blood agar supports the growth of most aerobic and anaerobic bacteria (vitamin K, cysteine and hemin supplementation enhances the growth of anaerobic bacteria) and fungi. Blood agar can indicate the degree of hemolysis caused by hemolysin. Based on this, it is used to differentiate

among gram-positive cocci. Hence, it is also known as a **differential medium.**

- **Beta hemolysis** refers to the complete lysis of the red blood cells and hemoglobin; this results in the complete clearing of the blood agar medium surrounding the colonies, e.g., group A streptococci (Fig. 4.1).

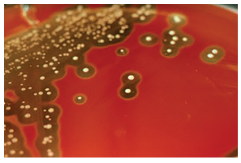


Fig. 4.1 Blood agar: *S. pyogenes* showing beta hemolysis (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- **Alpha hemolysis** refers to the partial lysis of red blood cells and hemoglobin; this results in a greenish discolouration of the blood agar around the colonies (Fig. 4.2), e.g., *Streptococcus pneumoniae.*

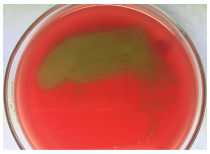


Fig. 4.2 Blood agar with alpha-hemolytic *Streptococcus pneumoniae* (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Chocolate agar This is made by heating a mixture of sheep blood and nutrient agar. This results in the release of hemoglobin, a related substance hemin (also called X factor) and nicotinamide adenine dinucleotide (NAD, also called V factor) during the process of heating. Chocolate agar is used to grow fastidious organisms, including *H. influenzae*, *N. meningitidis*, *N. gonorrhoeae* and *Pneumococcus* (Fig. 4.3).

Fig. 4.3 Chocolate agar with luxuriant growth (Source: Public Health Image Library, ID

12472 / Amanda Moore / CDC)

Brain–heart infusion broth (BHIB) This is a highly nutritious, buffered fluid culture medium prepared by non-enzymic infusion from calf brain and cow heart, often with added peptone and dextrose. It is suitable for the cultivation of fastidious organisms.

2. Enrichment media

These media are used to suppress commensal bacteria while allowing the pathogen to remain viable and to grow. It is employed for specimens with mixed flora, e.g., fecal sample from which diarrheagenic bacteria need to be isolated. Substances that have an inhibitory effect on the commensals are incorporated in the medium. Examples of enrichment media are **tetrathionate broth** and **selenite F broth**. Tetrathionate inhibits coliforms while allowing typhoid-paratyphoid bacilli to grow. Selenite F is used for fecal samples in cases of dysentery.

3. Selective media

As in the above case, if an inhibiting substance is added to a solid medium, it enables the required bacterium to form colonies while inhibiting other bacteria, e.g., **deoxycholate citrate agar (DCA)** for fecal samples and **thiosulphate citrate bile sucrose agar (TCBS)** for *Vibrio* species in cholera (Fig. 4.4). The alkaline pH of TCBS supports the growth of *Vibrio* (which cause cholera) while inhibiting other gut flora. Organisms that ferment sucrose appear yellow, while those that do not (*V. parahaemolyticus*) appear green; thus it also acts as an indicator medium.

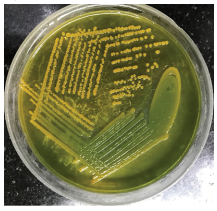


Fig. 4.4 TCBS agar with green colonies of *V. cholerae* (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Other selective media used for specific organisms are:

Thayer–Martin medium It contains antibiotics (vancomycin 3.0 mg, colistin 7.5 mg and nystatin 12.5 units per mL of agar) in chocolate agar. This is used to isolate *N. gonorrhoeae* from urethral and endocervical

swabs. The antimicrobials suppress the growth of commensal bacteria, which may inhibit the growth of *N. gonorrhoeae*. Thayer–Martin plates are incubated in an atmosphere containing 3–10% CO_2.

Lowenstein–Jensen medium This medium is used for the primary isolation of *Mycobacterium* species. It consists of mineral salts, asparagine, glycerol, malachite green and hen's eggs. The medium is sterilised by inspissation (the application of a temperature of 75°C for three consecutive days). Malachite green prevents the growth of most other microorganisms (Fig. 4.5).

Fig. 4.5 Lowenstein–Jensen's medium (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

4. Indicator media

These media contain an indicator that changes colour when a bacterium grows on it, e.g., **sulphite** in **Wilson–Blair medium**. *S. typhi* reduces sulphite to sulphide to give a black metallic sheen on the colony. **Potassium tellurite** in **McLeod's medium** (**potassium tellurite agar**) is reduced to metallic tellurium by *Corynebacterium diphtheriae* to produce black colonies (Fig. 4.6).

Fig. 4.6 Potassium tellurite agar (McLeod's medium–PTA) showing black colonies of *C. diphtheriae*

5. Differential media

The **MacConkey medium** consists of peptone, lactose, agar, neutral red and taurocholate and differentiates lactose fermenters as pink colonies,

while non-lactose fermenters appear colourless or pale. This may also be termed an **indicator medium**. Many facultative anaerobes in the intestine are lactose fermenters, which grow as pink colonies (*E. coli* and *Klebsiella*) (Fig. 4.7a and b). Several well-known pathogens do not ferment lactose (*Shigella* and *Salmonella* species) and are colourless (Fig. 4.7c).

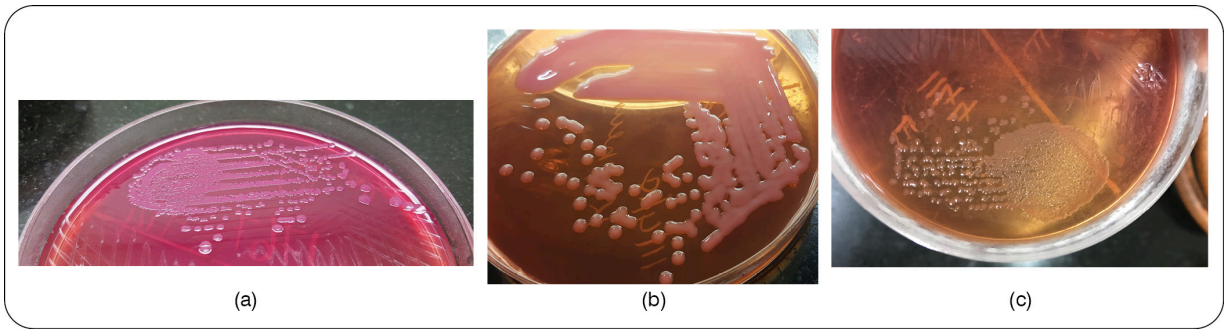


Fig. 4.7 MacConkey agar showing (a) flat, smooth, pink colonies of *Escherichia coli*; (b) large, mucoid colonies of *K. pneumoniae* and (c) smooth, colourless colonies of *Salmonella* (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Cysteine lactose electrolyte deficient (CLED) agar is another differential media used in the clinical laboratory for isolating and enumerating bacteria in urine (Fig. 4.8).

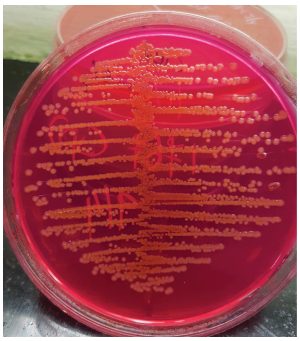


Fig. 4.8 CLED medium showing pink colonies of *Escherichia coli* isolated from urine (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

6. Transport media

Special media, termed transport media, are devised to transport specimens suspected to have fastidious organisms, e.g., **Stuart's transport medium**—a non-nutrient, soft agar gel containing a reducing agent (sodium thioglycolate) to prevent oxidation and charcoal to neutralise certain bacterial inhibitors—is used to transport specimens for the isolation of

gonococci; buffered glycerol saline is used for enteric bacilli. **Carey Blair** is another transport medium used for enteric pathogens.

7. Anaerobic media

These are used to cultivate anaerobic organisms like clostridia, *Bacteroides*, etc. These media contain reducing agents.

Anaerobic media include **Robertson's cooked meat medium** and **thioglycolate medium.** The reducing agents in these media—cooked meat and thioglycolate respectively—promote the growth of anaerobic organisms.

Robertson's cooked meat medium Growth of spore-forming and non-spore-forming obligate anaerobes is supported by this medium. Meat particles are added to enhance the growth of anaerobic microorganisms. Amino acids and other nutrients including reducing substances, which permit the growth of strict anaerobes, are supplied by the meat particles. Besides meat particles, the medium also contains dextrose, yeast extract, hemin and vitamin K.

Thioglycolate medium This medium supports the growth of all organisms with varied oxygen requirements: anaerobes, aerobes and facultative anaerobes. The medium is boiled before use to eliminate oxygen, which is less soluble at hot temperatures. Obligate aerobes grow only on the surface of the medium, microaerophiles in the middle, while anaerobes grow only at the bottom. The medium contains yeast extract, casitone, sodium chloride, *l*-cystine, thioglycolic acid, agar, methylene blue and deionised water at a final pH of 7.2.

8. Defined or synthetic media

These are media whose constituents are clearly defined in terms of each ingredient. They are chemically defined media that are prepared from pure chemical substances, e.g., minimum essential medium (used in tissue culture).

Some of the media commonly used in a clinical microbiology laboratory are presented in Table 4.2.

Table 4.2 Common media used in the clinical microbiology laboratory

Media	Specimen	Organisms

Nutrient agar/broth Peptone water	Not used for direct specimen inoculation	All non-fastidious bacteria Nutrient agar: To demonstrate pigment Broth: Used for culture to demonstrate motility
Blood agar	All aspirates, body fluids, blood, exudates, urine (except feces)	All common pathogenic bacteria (except *Hemophilus* and nutritionally deficient bacteria)
Chocolate agar	All samples	All common pathogenic bacteria
MacConkey agar	All samples including feces	All common gram-negative pathogens, *Enterococcus*
Deoxycholate citrate agar (DCA)	Feces	Enteric pathogens: *Salmonella, Shigella*
Cysteine lactose electrolyte deficient (CLED) agar	Urine	All urine pathogens
Thiosulphate citrate bile salt sucrose (TCBS) agar	Feces (suspected cholera)	*V. cholerae*
Potassium tellurite agar (PTA)	Throat swab (in suspected diphtheria)	*C. diphtheriae*
Robertson's cooked meat medium (RCM) / Thioglycolate	All samples for anaerobic pathogens (except feces)	For most anaerobic pathogens clostridia, *Bacteroides*

CULTURE METHODS

Bacteria need to be isolated in the laboratory for several reasons.

1. From clinical samples to establish etiology
2. To determine antibiotic susceptibility for appropriate antibiotic treatment
3. For studying virulence factors and for other research purposes
4. To study antigenic properties and antigen preparation
5. To estimate viable bacterial count
6. For pharmaceutical, industrial and agricultural purposes and utility

Inoculation Methods on Culture Media

Loops or straight wires made of platinum or nichrome (24 SWG) are used to transfer material on to culture plates or tubes. The loops are sterilised by

heating them over a flame until they are red-hot and then cooling them before using them to pick up specimen material. One loopful of the specimen is transferred onto the surface of a well-dried plate. The inoculum is then distributed thinly over the plate by streaking it with the loop in a series of parallel lines, in different segments of the plate.

The methods employed to inoculate the organisms on solid media are:
- Streak • Lawn
- Stroke • Stab
- Pour plate

Well-separated colonies are obtained over the final series of streaks (Fig. 4.9).

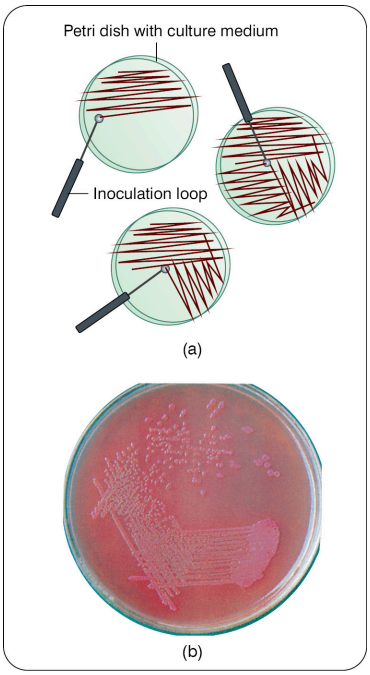


Fig. 4.9 Streak cultures to get isolated colonies

i) The **lawn** or **carpet culture** provides uniform surface growth of the bacterium and is useful for antibiotic sensitivity testing (disc method) or bacteriophage typing. It may also be employed when a large amount of growth is required for the preparation of bacterial antigens and vaccines.

ii) **Stab cultures** are prepared by puncturing a suitable medium with a long, straight, charged wire. Stab cultures are employed for demonstrating oxygen requirements. Bacteria that require oxygen grow on the surface while those that do not, grow at the bottom of

the stab. These cultures are also used in the maintenance of **stock cultures** and to demonstrate other properties like **gelatin liquefaction** on appropriate media.

iii) **Pour plate culture method** gives an estimate of the viable bacterial count in a suspension. It is done for quantitative urine cultures. Appropriate dilutions of the inoculum (of 1 mL) are added to the molten (40–45°C) agar, mixed well, poured into sterile petri dishes and allowed to set. After incubation, colonies are seen to be distributed throughout the depth of the medium. These can be counted using colony counters to give the exact colony count in 1 mL of urine or any other fluid.

Liquid cultures in tubes, bottles or flasks may be inoculated by touching them with a charged loop, pipette or syringe. Large inocula can be inoculated into liquid culture media. This is ideal for blood culture and sterility tests where a larger quantity of inoculum is required to isolate the organism. Liquid cultures are preferable for inocula containing antibiotics and other inhibitory substances as these are diluted out in the medium. Yield can be enhanced by agitation, aeration, addition of nutrients and removal of toxic metabolites (continuous culture methods). The major disadvantage of liquid culture is that it does not differentiate between organisms in mixed inocula.

Culture plates and tubes with liquid culture are incubated at 37°C in a **bacteriological incubator** (Fig. 4.10) overnight or longer before reading the plates/tubes to look for growth in the form of colonies (on solid media) or for turbidity.

Fig. 4.10 Bacteriological incubator set to a temperature of 37°C for optimal growth of most pathogenic bacteria (*Source:* Department of Microbiology, Pondicherry Institute of Medical

Anaerobic Culture Methods

Anaerobic bacteria differ in their requirements of and sensitivity to oxygen. Growth conditions may be different for strict anaerobes (*Clostridium tetani*) and aerotolerant bacteria (*Clostridium histolyticum)*.

Some of the commonly used methods of anaerobiosis are discussed in the following pages.

1. McIntosh and Filde's anaerobic jar

This is the most reliable and widely used anaerobic method (Fig. 4.11a and b). It consists of a stout glass or metal jar with a metal lid that can be clamped airtight with a screw. The lid has two tubes with taps, one acting as the gas inlet and the other as the outlet. The lid also has two terminals which can be connected to an electrical supply. Leading from the terminals and suspended by stout wires on the underside of the lid is a small, grooved, porcelain spool around which is wrapped a layer of palladinised asbestos. Inoculated culture plates are placed in the jar, and the lid is clamped shut. **Alumina pellets coated with palladium** and kept dry in a sachet within the jar, act as a catalyst at room temperature. An **indicator** is used for verifying the anaerobic condition in the jars. Reduced methylene blue is generally used for this purpose. It remains colourless anaerobically but turns blue on exposure to oxygen.

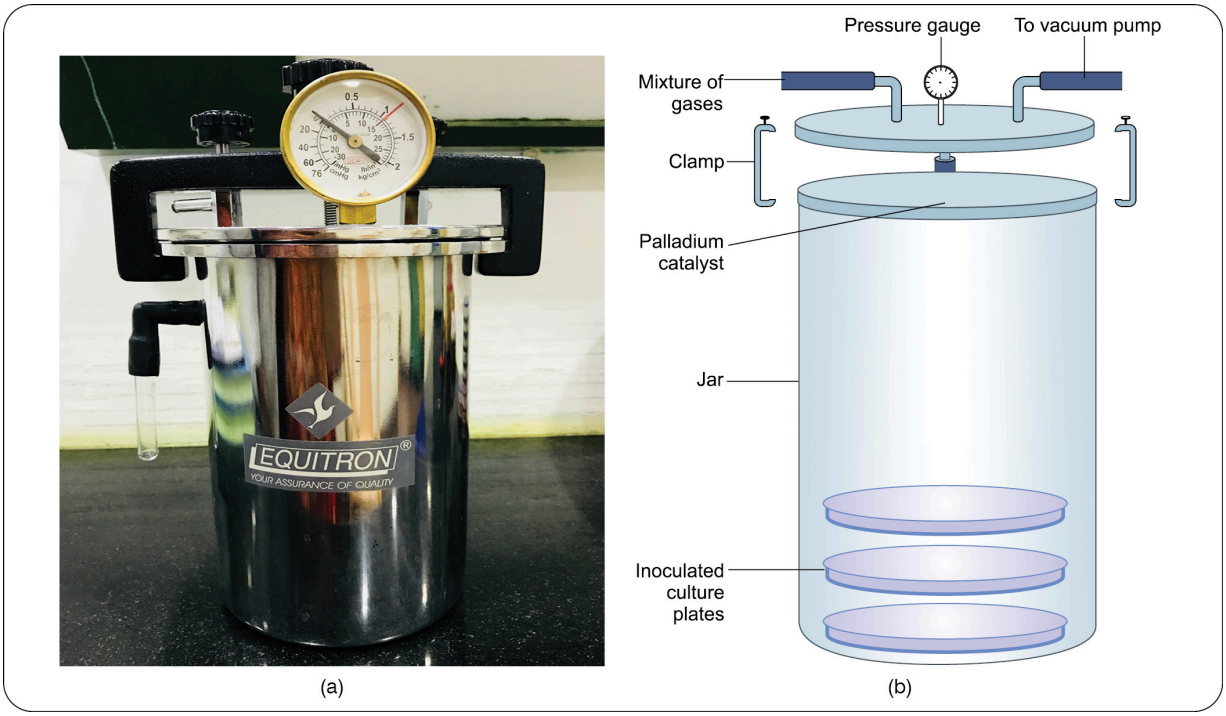


Fig. 4.11 McIntosh and Filde's anaerobic jar: a) a photograph and b) diagrammatic representation (*Source:* (a) Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

2. Anoxomat

This is an automated, microprocessor-controlled system for the cultivation of anaerobic, microaerophilic and capnophilic bacteria. It uses the evacuation and replacement method developed by McIntosh and Filde to create an oxygen-free environment for anaerobic bacteria.

This method is the most efficient means of bacterial cultivation. The system creates an environmental condition that can be changed for clinical, pharmaceutical and other microbiological uses.

3. Gaspak

This is the method of choice for anaerobiosis. The Gaspak (Fig. 4.12) is commercially available as a disposable envelope, containing chemicals which generate hydrogen and carbon dioxide on addition of water. After the inoculated plates are kept in the jar, the Gaspak envelope, with water added, is placed inside and the lid screwed tight. Hydrogen and carbon dioxide are liberated in the presence of a cold catalyst in the envelope. This permits the combination of hydrogen and oxygen to produce an anaerobic environment. The Gaspak is simple and effective, eliminating the need for creating a

vacuum and adding hydrogen.

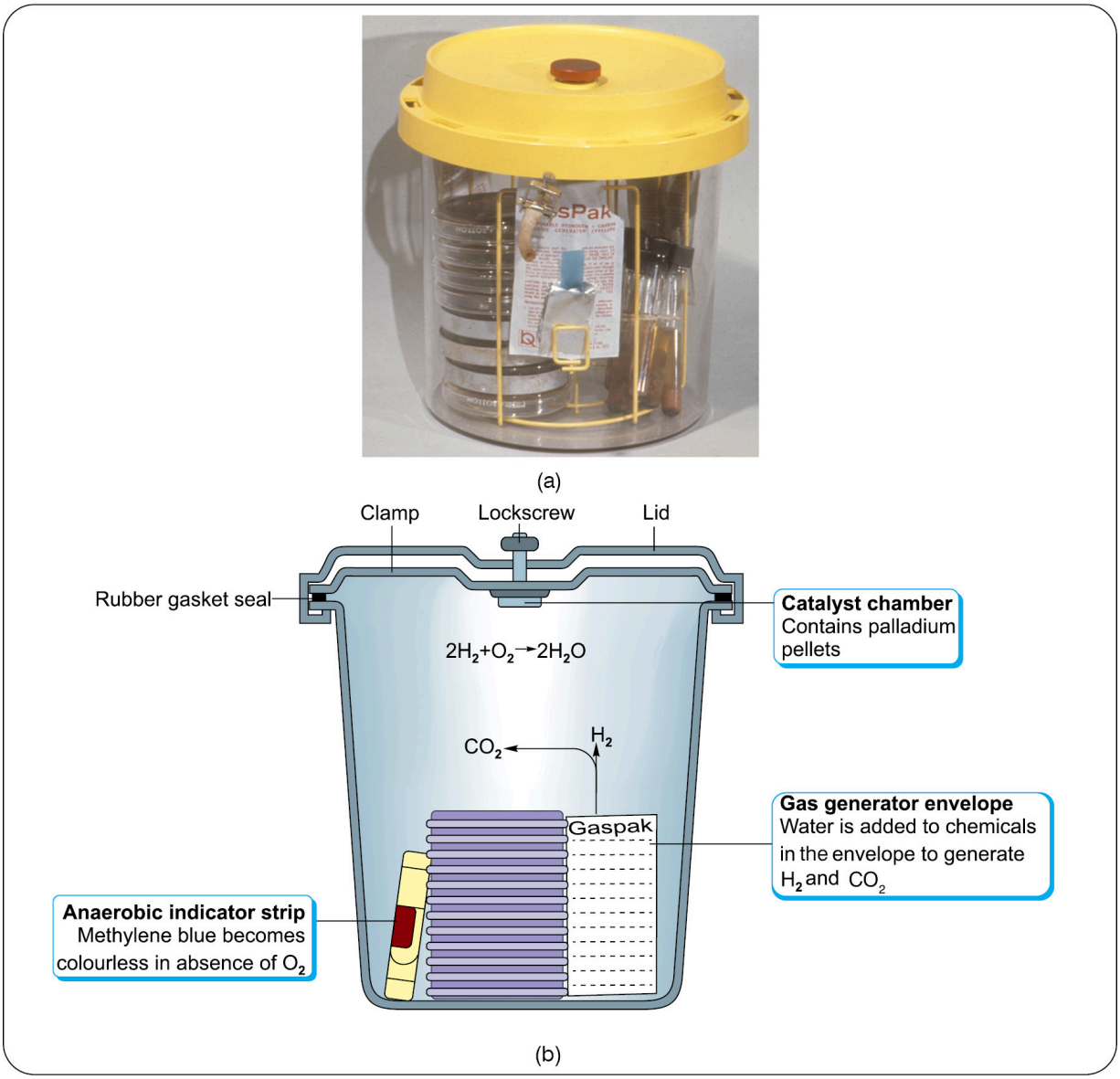


Fig. 4.12 Gaspak: (a) typical Gaspak air-tight anaerobic jar and (b) line diagram of anaerobic jar (*Source:* (a) Public Health Image Library, ID 3595 / Dr. Gilda Jones / CDC)

4. Pre-reduced anaerobic system (PRAS)

Pre-reduced media are commercially available for fastidious anaerobes.

5. Anaerobic chamber ('glove box')

The anaerobic chamber is an airtight, glass-fronted cabinet filled with inert gas, with an entry lock for the introduction and removal of materials, and gloves for the hands.

6. Other methods of anaerobiosis

- **Robertson's cooked meat (RCM) medium** is probably the most widely used fluid medium for the culture of anaerobes. It consists of fat-free, minced cooked meat in broth (Fig. 4.13). It permits the growth of even strict anaerobes and indicates their saccharolytic or proteolytic activities by the meat being turned red or black, respectively.

Fig. 4.13 Robertson's cooked meat medium—widely used medium for transport and culture of anaerobic organisms (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- **Thioglycolate broth** with hemin and vitamin K is an enriched liquid medium for culturing anaerobic and microaerophilic bacteria (Fig. 4.14).

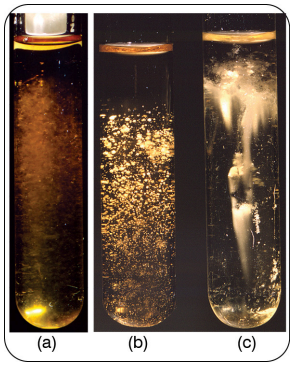


Fig. 4.14 Growth in thioglycolate broth: (a) uniform turbidity; (b) granular turbidity and (c) surface growth (*Source:* (a) Public Health Image Library, ID 19510 / CDC and (b and c) Public Health Image Library, ID 11980 / Dr. Lucille Georg / CDC)

- **Anaerobic broth** is an easily prepared anerobic medium. Smith–Noguchi medium is used to cultivate Reiter's strain of *Treponema*. It is an anaerobic medium containing fresh animal tissue, such as rabbit kidney, spleen, testes or heart.
- **Reducing agents** are used to achieve the reduction of oxygen in the medium. The frequently used reducing agents include 1% glucose, 0.1% thioglycolate, 0.1% ascorbic acid and 0.05% cysteine.

The addition of a small quantity of agar enhances the anaerobic capacity of the medium by slowing the diffusion of oxygen in it.

Methods of Isolating Pure Cultures

- **Surface plating** is the method routinely employed in clinical bacteriology and enables the isolation of distinct colonies.
- **Enrichment, selective** and **indicator media** are widely used for the isolation of pathogens from specimens with varied flora, e.g., feces.
- Pure cultures may be obtained by the **pre-treatment of specimens** with appropriate bactericidal substances which destroy the commensal bacteria. Tubercle bacilli are isolated from sputum and other clinical specimens by treating the material with alkali, acid or other substances to which most commensals are susceptible but tubercle bacilli are resistant.
- Separation of bacteria with different optimum temperatures can be effected by **incubation at different temperatures.** Only thermophilic bacteria grow at 60°C. A mixture containing *N. meningitidis* and *N. catarrhalis* can be purified by incubation at 22°C, at which temperature, only the latter grows.
- By **heating** a mixture containing vegetative and spore-forming bacteria at 80°C, the former can be eliminated. This method is useful for the isolation of tetanus bacilli from dust and similar sources.
- **Craigie's tube** is used for the separation of motile from non-motile bacteria. This consists of a tube of semisolid agar, with a

narrow tube open at both ends. It is placed in the centre of the medium, with one end projecting above the level of the medium. The mixture is inoculated into the central tube. On incubation, the motile bacteria alone traverse the agar and are recovered from the surface of the medium outside the central tube. A U-tube also serves the same purpose. This method is used to obtain phase variants in motile bacteria.

- **Inoculation into susceptible animals** can be used to isolate pathogenic bacteria from mixtures. Anthrax bacilli can be distinguished from other aerobic sporulating bacilli by inoculating the bacteria into mice or guinea pigs. They produce fatal septicemia and can be cultured pure from heart blood. Bacteria of differing sizes may be separated by **selective filters** (used to separate viruses from bacteria).

Preservation of Microorganisms

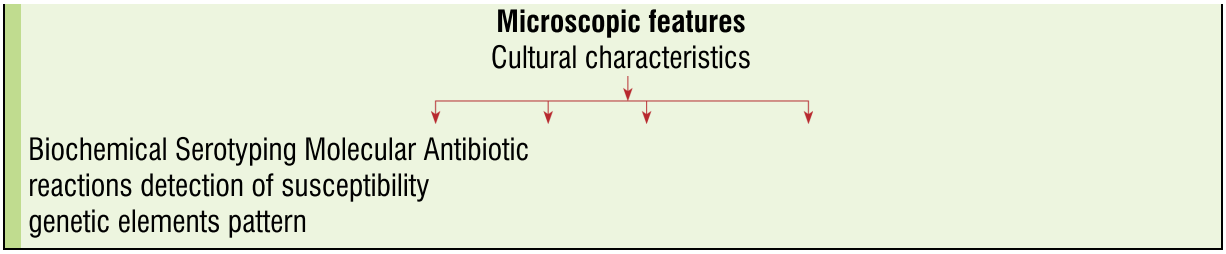
Bacteria and viruses can be preserved by the following methods:

- Freezing at −80°C or in liquid nitrogen at a temperature of 195.79°C (−320°F)
- Lyophilisation—rapid freezing and thawing using a lyophiliser
- Subculturing on solid/liquid media for short-duration viability media
- In glycerol stock (80% v/v) at –70°C

IDENTIFICATION OF BACTERIA

Once a bacterium is obtained in pure culture, it must be identified. A systematic, stepwise procedure is adopted in a clinical microbiology laboratory for the identification and susceptibility testing of bacteria. Several automated systems have become useful in hastening the process of isolation and confirmation of clinical isolates.

Steps in identification The morphology of bacteria is determined by staining, followed by cultural characteristics, biochemical tests, confirmation by immunological methods followed by testing for pathogenicity (virulence) in some instances. Molecular detection and confirmation by nucleic acid-based platforms are recent advances in diagnosis.



The following methods based on bacterial characteristics are studied for identification:

1. Morphology

The morphology of a bacterium depends on a number of factors such as the strain studied, nature of the culture medium, temperature and time of incubation, age of the culture and the number of subcultures it has undergone. This is studied best by microscopy after staining the bacteria.

The **characteristics** noted are shape, size, flagella, spores and capsules and arrangement of the group.

- ***Shape*** Bacteria may be spherical, filamentous, rod-shaped, comma-shaped or a spiral. The axis of the organism may be straight or curved.
- ***Size*** The length and breadth of bacteria may vary. Its sides may be parallel, convex, concave or irregular. The ends may be cut straight, rounded or tapering. Considerable variation in shape and size leading to club, navicular and swollen or shadow or giant forms may be seen.
- ***Arrangement*** Bacteria may be arranged singly, in pairs, in tetrads, in packets of eight, or in chains, short or long, in the case of cocci; bacilli may be arranged at random, in short or long chains, in Chinese letter patterns, as palisades or in bundles; vibrios may be single or in S or spiral forms (Fig. 4.15).

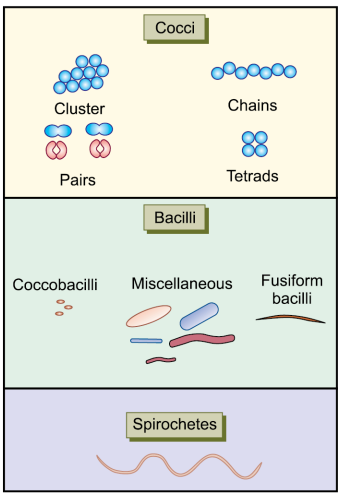


Fig. 4.15 Examples of common bacterial cellular morphologies

- ***Flagella*** Bacteria may be without **flagella**, that is, atrichate, or monotrichate, lophotrichate, amphitrichate or peritrichate. Flagella cannot be visualised by a light microscope unless stained by special methods.
- ***Spores*** These, when present, may be oval, spherical or ellipsoidal and may be of the same width or wider than the bacillary body. The spores may be equatorial, subterminal or terminal.
- ***Capsules*** These may or may not be present. They can be demonstrated by negative staining, dark-ground illumination, phase-contrast or electron microscopy.

Motility

Bacteria may be non-motile, sluggishly motile, actively motile or may exhibit darting motility. Motility is seen best by the hanging drop method when the medium is liquid (nutrient broth) or as diffuse growth in a semisolid medium.

Staining reactions

The age of the culture is important. In older cultures, staining characteristics either vary or are not brought out well. Simple stains bring out the morphology best. Differential and special stains are necessary to study characteristics like flagella, capsules, spores and metachromatic granules. **Gram stain** divides bacteria into the gram-positive and the gram-negative

while **Ziehl–Neelsen stain** divides it into acid-fast and non-acid-fast. The **fluorescent antibody technique** enables identification by surface antigens.

The study of morphology and staining characteristics helps in the preliminary identification of the isolate (Figs. 4.16–4.18).

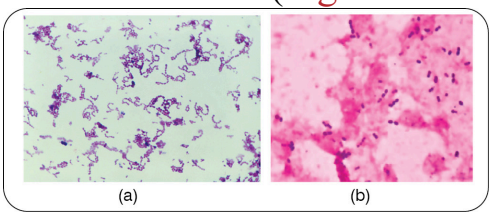


Fig. 4.16 Gram stain of a pus smear. Gram-positive, violet-coloured cocci: (a) in short chains and (b) in pairs, with pus cells stained pink (*Source:* (a) Public Health Image Library, ID 4347 / Dr. Gilda Jones / CDC and (b) Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

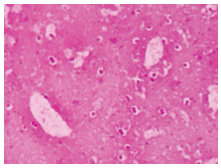


Fig. 4.17 Gonococci in urethral discharge. Gram stain showing gram-negative diplococci (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

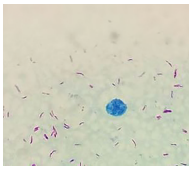


Fig. 4.18 Acid-fast stain (Ziehl–Neelsen stain) of sputum; *M. tuberculosis* are seen as pink rods (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

2. Cultural Characteristics

These provide additional information for the identification of the bacterium. The characteristics revealed in different types of media are noted.

Solid media

The following colony characteristics are noted on solid media (Fig. 4.19):

- **Shape**—circular, irregular or rhizoid
- **Size** in millimetres
- **Elevation**—effuse, elevated, convex, concave, umbonate or umbilicate

- **Margins**—bevelled or otherwise
- **Surface**—smooth, wavy, rough, granular, papillate or glistening
- **Edges**—entire, undulate, crenated, fimbriate or curled
- **Colour**—golden-yellow, white, buff, pink, etc.
- **Structure**—opaque, translucent or transparent
- **Consistency**—membranous, friable, butyrous or viscid
- **Emulsifiability**

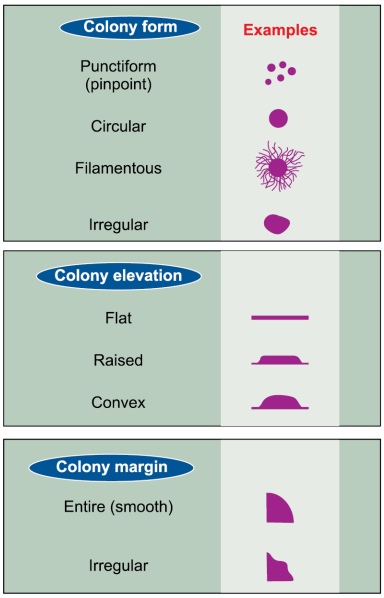


Fig. 4.19 Colony morphology showing different forms, elevation and margin of bacterial colonies

Liquid media

In **liquid culture**, the following characteristics are noted:

- Quantum of growth
- Turbidity
- Presence of deposit and its character
- Nature of surface growth such as pellicle

3. Biochemical Tests

The tests based on fermentation of carbohydrates and other biochemical properties are used for the characterisation of bacteria.

Some important and commonly used tests are described below:

i. Carbohydrate fermentation

This is tested in media containing a single carbohydrate (sugar).

The term 'sugar' in microbiology denotes any fermentable substance. They may be:
- **Monosaccharides**
  – pentoses, e.g., arabinose, xylose
  – hexoses, e.g., dextrose, mannose
- **Disaccharides**, e.g., saccharose, lactose
- **Trisaccharides**, e.g., raffinose
- **Polysaccharides**, e.g., starch, inulin
- **Alcohols**, e.g., glycerol, sorbitol
- **Glucosides**, e.g., salicin, esculin
- **Non-carbohydrate** substances, e.g., inositol

The usual sugar media consist of 1% of the sugar in peptone water along with an appropriate indicator. A small tube (Durham's tube) is kept inverted in the sugar media to detect the production of gas. Acid production is shown by a change in the colour of the medium to pink or red depending on the indicator used. Gas produced is indicated by a bubble in the Durham's tube.

For organisms that are exacting in their growth requirements (e.g., *N. gonorrhoeae*), **Hiss' serum sugars** (containing 3% serum along with the carbohydrate) are used.

ii. Indole production

A culture of the organism in peptone water following 48 or 96 hours of incubation at 37°C is tested for indole production. This test demonstrates the production of indole from tryptophan. **Kovac's reagent** (0.5 mL) is added to the culture and shaken gently.

- **Indole-positive** A red colour ring indicates a positive reaction (e.g., *E. coli*) (Fig. 4.20)

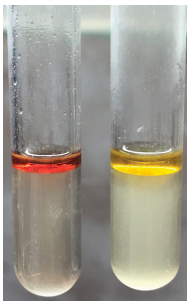


Fig. 4.20 Indole production test; red colour is positive for indole and yellow is negative (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- **Indole-negative** A yellow colour ring formed at the surface of the broth indicates a negative reaction

iii. Methyl red test (MR)

This test is used to detect the production of acid during the fermentation of glucose and the maintenance of **pH below 4.5** in an old culture. Five drops of 0.04% solution of methyl red are added to the culture in glucose phosphate medium which had been incubated at 30°C for five days. The test is read immediately after mixing the indicator.

MR-positive Red colour denotes positive (e.g., *E. coli*)

MR-negative Yellow signifies a negative test

iv. Voges–Proskauer test (VP)

This demonstrates the production of **acetyl methyl carbinol** from pyruvic acid as an intermediate stage in its conversion to **2:3 butyleneglycol**. In the presence of alkali and atmospheric oxygen, the small amount of acetyl methyl carbinol is oxidised to **diacetyl,** which reacts with the peptone of the broth to produce a red colour.

The test is performed by adding 0.6 mL of a 5% solution of α-naphthol in ethanol and 0.2 mL of 40% KOH to 1 mL of a glucose phosphate medium culture of the organism incubated at 30°C for five days or 37°C for 48 hours.

VP-positive In a positive reaction, a pink colour appears in 2–5 minutes, deepening to magenta or crimson in half an hour (e.g., *Klebsiella*).

VP-negative In a negative reaction, it remains colourless for half an hour.

Traces of pink colouration should be ignored.

v. Citrate utilisation

The utilisation of citrate as a sole source of carbon (as is the case with *Citrobacter*) is shown by **Koser's citrate medium** by the production of turbidity in the medium. On the other hand, **Simmon's citrate** (solid medium) changes to blue colour to indicate growth (Fig. 4.21).

Fig. 4.21 Simmon's citrate test; blue indicates positive and green is negative for citrate utilisation (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Indole, MR, VP and citrate tests are useful in the identification and classification of enteric gram-negative bacteria. These tests are commonly referred to as '**IMViC**' tests.

vi. Nitrate reduction (NR)

Bacteria grown for five days at 37°C in a broth containing 1% KNO_3 are tested for nitrate reduction. The test reagent consists of a mixture of equal volumes of sulphanilic acid and α-naphthylamine in 5N acetic acid, mixed just before use. 0.1 mL of the test reagent is added to the culture. This is a test for the presence of the enzyme **nitrate reductase** which reduces nitrate to nitrite (e.g., Enterobacteriaceae).

NR-positive A red colour within a few minutes signifies a positive reaction.

NR-negative Absence of colour indicates a negative reaction.

vii. Urease test

This test is done in **Christensen's urease medium**. The slope is heavily inoculated and incubated at 37°C. It is examined after four hours and after overnight incubation. The test should not be considered negative till after four days of incubation.

Urease-positive These cultures (e.g., *Proteus*) produce a purple-pink

colour. Urease-producing bacteria reduce urea to ammonia, which is responsible for the change in the colour of the medium (Fig. 4.22).

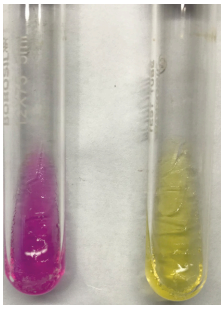


Fig. 4.22 Urease production is indicated by pink colour; yellow indicates negative test. (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Urease-negative These cultures produce a yellow colour.

viii. Hydrogen sulphide production

Some organisms break down amino acids like cysteine to release H_2S during protein degradation.

The H_2S reacts with iron in the medium (in which the bacteria is grown) to produce black precipitates of ferric sulphide (e.g., *Proteus mirabilis*).

This is demonstrated in composite media like **triple sugar iron (TSI) agar** (Fig. 4.23) or **Kligler iron agar (KIA).**

Fig. 4.23 Blackening of the medium indicating H_2S production in TSI medium (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

ix. Catalase production

Catalase enzyme, which is produced by some organisms, can be used as an identifying character. Colonies from nutrient agar are picked up with a glass rod and placed in a tube containing 0.5 mL of 3% H_2O_2.

Prompt effervescence indicates catalase production (e.g., staphylococci) (Fig. 4.24). Culture media containing blood are unsuitable for the test as

blood contains catalase.

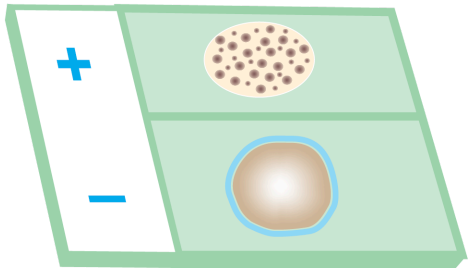


Fig. 4.24 Catalase test: the upper slide shows effervescence, while the drop in the lower slide is negative

x. Oxidase reaction

Cytochrome oxidase enzyme produced by aerobic organisms catalyses oxidation of reduced cytochrome by oxygen. A 1.0–1.5% solution of tetramethyl *p*-phenylene diamine hydrochloride (oxidase reagent) is poured over the colonies. Oxidase-positive colonies become purple or black in 30 seconds to 1 minute. A filter paper soaked in the oxidase reagent is smeared with the colony.

Oxidase-positive reaction In a positive test, the smeared area turns dark purple in 10 seconds (e.g., *Pseudomonas aeruginosa*). The solution should be freshly prepared.

xi. Lecithinase production (on egg yolk agar)

Organisms producing lecithinase (e.g., *C. perfringens*), when grown on a solid egg yolk agar, form colonies surrounded by a zone of clearing. This is because lecithin of the egg gets digested by lecithinase produced by the organism.

xii. Litmus milk test

Either there is no change in the medium or acid or alkali may be produced. Clotting of milk, peptonisation or saponification may occur. The clot may be disrupted by the gas produced **(stormy fermentation).**

xiii. Growth in the presence of KCN

Buffered liquid medium containing potassium cyanide (KCN) in a final concentration of about 1/13,000 is used to identify some KCN-tolerant enteric bacilli (e.g., *E. coli*).

xiv. Composite media for biochemicals

A single composite medium indicates different properties of the bacterium that otherwise would have required the use of many separate media. A popular composite medium is the **triple sugar iron (TSI) medium.** A bacterium may ferment glucose only, or lactose and sucrose also, with or without gas formation which is indicated by the change in colour of the slant, slant and butt, or no change in colour. This medium may also indicate H_2S production. Some combinations are mentioned below.

TSI reactions may be one of the following (Fig. 4.25a–d):

i) Alkaline slant/acid butt with or without gas
ii) Acid slant/acid butt with gas
iii) Alkaline or acid slant/acid butt with H_2S/with or without gas
iv) Alkaline slant/alkaline butt may have H_2S
v) No change, meaning non-fermenter

(a) (b) (c) (d)

Fig. 4.25 Triple sugar iron (TSI) agar test: (a) alkaline slant and acid butt without any gas or H_2S (K/A^- −); (b) acid slant by acid butt with gas but no H_2S (A/A^+ −); (c) alkaline slant by acid butt with no gas with abundant H_2S (K/A^- +) and (d) alkaline slant by alkaline butt, no acid or H_2S (K/K^- −) (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Bile esculin medium is used to differentiate between *Enterococcus* (group D) from other streptococci (e.g., *S. mitis*). Characteristics demonstrated are the growth in the presence of 40% bile and the ability to hydrolyse esculin to esculetin and glucose. Esculetin combines with ferric ions to produce a black complex (phenolic iron complex).

xv. Other tests

- Tests such as fermentation of organic acids, oxidation of gluconate, amino acid decarboxylation, and hydrolysis of sodium hippurate are some of the other tests that are employed to characterise some bacteria.
- Resistance to heat and disinfectants is another characteristic (e.g., *E. faecalis* is resistant to heat at 60°C for half an hour while clostridial spores are resistant to boiling for various periods).
- Resistance to antibiotic and chemotherapeutic agents and bacteriocins would also help in differentiation and identification.

4. Immunological Methods

These are based on the surface antigenic structure of bacteria. Serotypes or serovars are detected by using specific antisera. Methods are based on agglutination or other suitable serological reactions. Immunofluorescence test is useful in some cases.

5. Pathogenicity Tests in Laboratory Animals

Often, it becomes necessary to perform in vivo tests to demonstrate the production of toxins or other virulence characters. Pathogenicity/virulence tests in an organism are done by inoculation into laboratory animals like the guinea pig, rabbit, rat and mouse. Routes of inoculation are:

- Intradermal
- Subcutaneous
- Intramuscular
- Intraperitoneal
- Intracerebral
- Intravenous
- Oral or nasal spray

These tests were performed commonly in the past. However, they are rarely used now because simpler in vitro tests in tissue culture are available and also due to stringent animal ethics guidelines.

6. Automated Systems

With the advances in technology and increasing use of computers in microbiology, the use of automation is slowly replacing the conventional methods of identification. Automated systems have the advantage of a rapid turnaround time and uniformity in the methods. But since they are based on a database, there is a need to upgrade the database as more information gets generated.

7. Nucleic Acid-Based Identification

These systems identify bacteria by recognising specific DNA sequences. This is done by direct detection of the target gene by microarrays or **real-time polymerase chain reaction** (RT-PCR) (Fig. 4.26) or **sequence-based gene amplification methods** (principle and details in Part IV).

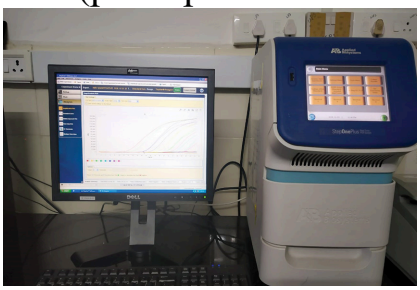


Fig. 4.26 Real-time PCR (*Source:* Pushpagiri Institute of Medical Sciences and Research Centre, Thiruvalla, Kerala)

8. MALDI-TOF

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation-Time of Flight) (Fig. 4.27) employs mass spectrometry, in which identification is based on the unique protein composition of bacterial cells. MALDI-TOF is being increasingly used in laboratories with heavy loads of samples for decreasing the turnaround time, to identify bacteria and to analyse the microorganisms in the environment and in the pharmaceutical, veterinary and food industries.

Fig. 4.27 MALDI-TOF MS—for rapid identification of bacteria (*Source:* Apollo Speciality Hospitals, Chennai, Tamil Nadu)

Applications in bacteriology

Detection of bacterial growth The automated systems used for the detection of bacterial growth are continuous monitoring systems and used mainly for detecting bacteria in cultures of blood and body fluids. They detect the presence of bacteria based on the production of CO_2 as an end product of the metabolism of bacteria. The presence of CO_2 is detected by sensors which transmit the readings to computers for analysis. With the first evidence of growth, the bottles are flagged (by a beep). They are then removed from the system and a smear is done to see the type of organism. A rapid smear report is then conveyed to the clinician for starting specific antibiotics for gram-positive or gram-negative organisms. Examples of available commercial systems are **BacT/Alert** (Fig. 4.28a and b), **BACTEC**, etc.

Identification systems The automated methods used for identifying bacteria are based on their biochemical characteristics or metabolic properties. The pattern is analysed based on the database in the computer software. They also additionally have methods for performing antimicrobial susceptibility tests. The common commercial systems available at present are **VITEK** (Fig. 4.29), **Phoenix, Auto Microbic System and Alfred 60 System.**

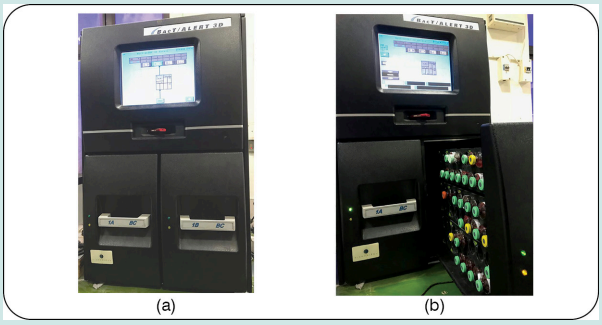


Fig. 4.28 (a) Automated blood culture system and (b) slots with bottles; with the first evidence of growth, the bottles are flagged (by a beep) (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Fig. 4.29 VITEK rapid bacterial identification system (*Source:* Apollo Speciality Hospitals, Chennai, Tamil Nadu)

The conventional method of identification of bacteria in a clinical microbiology laboratory follows a simple algorithm to identify common pathogens causing infections. ***Example:***

Microscopy—To detect the morphology	
Characterisation of GNB (gram-negative bacilli)	**Characterisation of GPC (gram-positive cocci)**
• Motility (Hanging drop)	• Coagulase (staphylococci)
• Carbohydrate fermentation (TSI or carbohydrates, etc.)	• Sensitive to bacitracin (streptococci)
• Enzyme production (indole, oxidase, urease, etc.)	• Sensitive to optochin (pneumococci)
• Ability to grow in single substrate medium (citrate)	• Growth in bile esculin (enterococci)
• Deamination of amino acids (lysine, arginine, etc.) • Serotyping/Serogrouping using specific antisera for confirmation (*Salmonella, Shigella,* streptococci, etc.)	• Antibiotic susceptibility on appropriate media [Mueller–Hinton agar (MHA), or MH blood agar for fastidious organisms] for both gram-negative and gram-positive bacteria.

Key points

- Culture media can be categorised as solid and liquid. Solid media are generally produced in the form of a gel by the addition of agar but sometimes they may be formed with heated serum or egg.
- They may be enriched, enrichment, selective, indicator, differential, sugar, transport or anaerobic types.
- Enriched media are used for bacteria that have exacting nutritional needs, for example, brain–heart infusion broth, blood agar, chocolate agar and others.
- Enrichment media are liquid media to which substances that have a stimulating effect on the bacteria to be grown or an inhibitory effect on those to be suppressed are incorporated in the medium, for example, tetrathionate broth and selenite F broth.
- Selective media are solid media to which an inhibiting substance is added, for example, Thayer–Martin medium, desoxycholate citrate medium and others.
- An indicator medium changes colour when a certain type of bacterium grows in it, for example, Wilson–Blair medium.
- A differential medium helps to differentiate characteristics of bacteria, for example, MacConkey medium.
- A transport medium is used for the transport of samples containing delicate organisms.
- Culture methods are used in the laboratory to isolate bacteria in pure culture and demonstrate their properties.

- The methods of inoculation include streak, lawn, stroke, stab, pour plate and liquid cultures.
- Aerobic bacteria require oxygen for growth and may be obligate aerobes or facultative.
- Anaerobic bacteria grow in the absence of oxygen; obligate anaerobes may even die on exposure to oxygen.
- Microaerophilic bacteria are those that grow best in the presence of low oxygen tension.
- Anaerobic culture methods include exclusion or displacement of oxygen with other gases, production of vacuum, absorption of oxygen by chemical or biological means and reduction of oxygen.
- McIntosh Filde's anaerobic jar is a widely used anaerobic method.
- Gaspak is a commercially available method for creating an anaerobic atmosphere.
- The culture media used for anaerobic culture include thioglycollate broth, Robertson's cooked meat medium and Smith-Noguchi medium.
- For fastidious anaerobes, pre-reduced media and anaerobic chambers are used.
- Pure culture of bacteria from mixtures is obtained by surface plating; enrichment, selective and indicator media; pre-treatment with appropriate bactericidal substances; separation of bacteria by incubation at different temperatures; and separation of motile and non-motile bacteria by using Craigie's tube.
- Pathogenic bacteria may be purified from mixtures by inoculation into appropriate animals.
- Bacteria of different sizes can be separated by the use of selective filters.
- The approaches to the identification of bacteria include morphology of bacterial colony on culture and detection of bacterial products released during metabolism.
- Techniques for direct microscopic examination of specimens include wet mounts (wet films) and stained preparations (Gram).
- Culture techniques where bacteria are isolated in pure culture are generally necessary because bacteria may be present in numbers too small to be seen by direct microscopy. Moreover, if bacteria are isolated, they can be precisely identified and antibacterial susceptibility tests can be run.
- Automated systems (like BactT, VITEK, etc.) are replacing conventional methods for identification of bacteria. They identify bacteria by computer-based software.
- Molecular methods for detection by microarray or real-time PCR or sequencing can be used.
- MALDI-TOF is an identification system based on the unique protein composition of bacterial cell.

Questions

Essays

1. Mention the scheme for isolating and identifying a clinical isolate in the laboratory.

2. Classify media. Mention their uses in the laboratory.
3. List the properties of indicator media and mention their specific use.

Short Answers

1. Selective media
2. Anaerobic culture methods
3. Methods of isolating pure cultures of bacteria
4. Methods for identifying bacteria isolated on a culture plate

CHAPTER 5

Genetics of Bacteria

Chapter navigation

- Principles of bacterial genetics
- Gene transfer, including transfer of antibiotic resistance
- Application of molecular genetics

INTRODUCTION

Bacteria, like all other organisms, obey the laws of genetics. It was in the 1940s that the principles of genetics were applied to bacteria and their viruses. This led to the birth of a new branch of science—**molecular biology**—which has wide applications ranging from understanding the genetic mechanisms of bacterial virulence, survival and resistance to antibiotics to the detection of hitherto unknown microorganisms. Molecular techniques now play an important role in rapid diagnosis and in producing genetically engineered vaccines for disease prevention.

BASIC PRINCIPLES OF MOLECULAR BIOLOGY

The central dogma of molecular biology rests on the following principle: Deoxyribonucleic acid (DNA) carries genetic information, which is transcribed onto ribonucleic acid (RNA) and then translated into the particular polypeptide
(DNA → RNA → polypeptide). As the nature and functions of a cell are basically determined by the specific polypeptides that constitute its proteins and enzymes, it follows that the essential material of heredity is the DNA. This is the storehouse of all information required for protein synthesis. An exception exists in the case of some viruses in which the genetic material is RNA instead of DNA (*this is discussed in the section on virology.*)

Structure of DNA

The DNA molecule is composed of two chains of nucleotides wound together in the form of a '**double helix**'. Each chain has a backbone of **deoxyribose** and **phosphate residues** arranged alternately. Attached to each deoxyribose is one of four nitrogenous bases:

- **Adenine** (A) } **Purines**
- **Guanine** (G)
- **Thymine** (T) } **Pyrimidines**
- **Cytosine** (C)

The double-stranded nature of the molecule is stabilised by hydrogen bonding between the bases on opposite strands in such a way that adenine is always linked to thymine, and guanine to cytosine (Fig. 5.1).

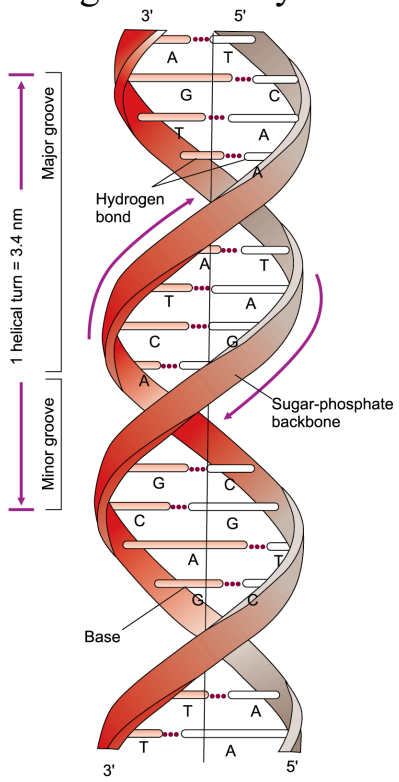


Fig. 5.1 A schematic drawing of the Watson–Crick's structure of DNA showing helical sugar–phosphate backbone of the two strands held together by hydrogen bonding between the bases

Adenine and thymine thus form a complementary base pair, and guanine and cytosine form another. A molecule of DNA will, therefore, contain as many units of adenine as thymine, and of guanine as cytosine. However, the ratio of each pair of bases (A + T)/(G + C), though constant for each species, varies widely from one bacterial species to another. **Bacteria are grouped based on their G–C content.** The DNA molecule replicates by first unwinding at one end to form a fork, each strand of the fork acting as a template for the synthesis of a complementary strand, with which it then forms a double helix (Fig. 5.2).

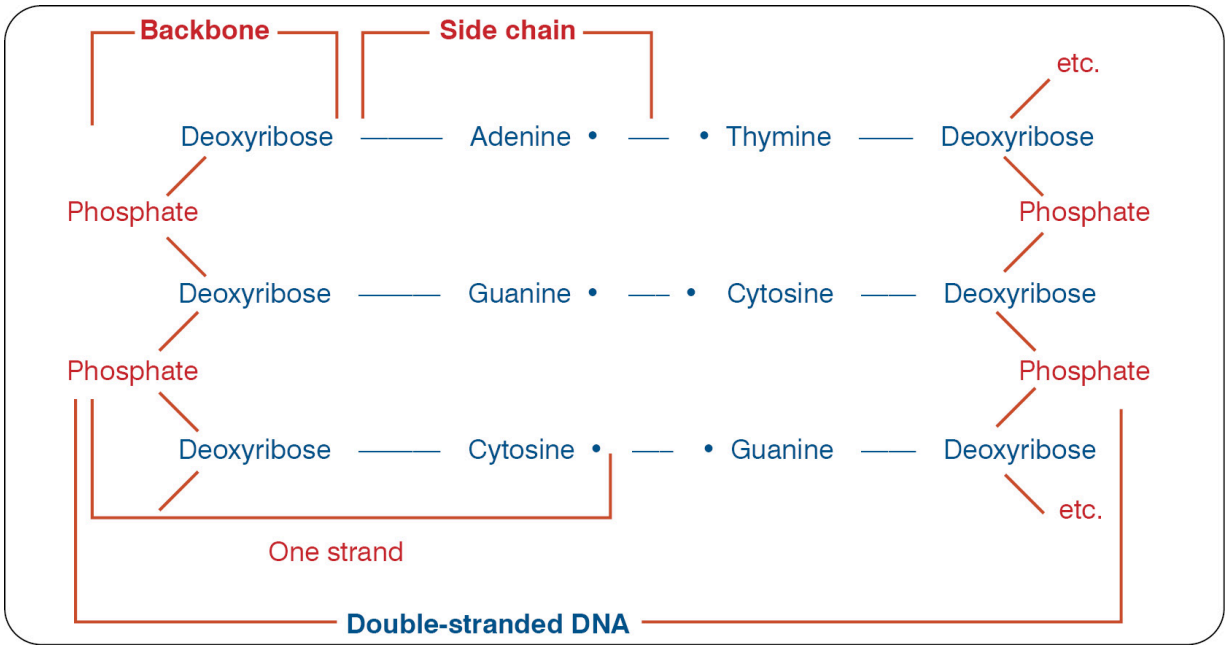


Fig. 5.2 A segment of double-stranded DNA illustrating its chemical structure

Structure of RNA

RNA is structurally similar to DNA, except for two major differences. It contains the **sugar ribose** (instead of deoxyribose which is present in DNA) and the **base uracil** (instead of thymine, which is present in DNA).

Types of RNA

Three distinct types of RNA can be distinguished on the basis of structure and function:

1. **Messenger RNA (mRNA)**
2. **Ribosomal RNA (rRNA)**

3. **Transfer RNA (tRNA)**

DNA acts as the template for the synthesis of mRNA and, therefore, the bases in the two will be complementary to each other. Adenine, guanine, cytosine and uracil in mRNA will be complementary to thymine, cytosine, guanine and adenine, respectively, in DNA.

TERMS RELATED TO GENETICS

Codon

- ***Sense codon*** Genetic information is stored in the DNA as a code, consisting of a **sequence of three bases**. Each triplet (codon) transcribed on mRNA specifies for a single amino acid. More than one codon may exist for the same amino acid.
    - The **start codon** or the initiator codon is the first **codon** of a messenger RNA (mRNA) transcript that is translated by a ribosome.
- ***Nonsense codons*** Three codons **(UAA, UAG and UGA)** do not code for any amino acid and are called 'nonsense codons'. They act as punctuation marks (**stop codons**), terminating the message for the synthesis of a polypeptide.
- ***Anticodons*** These are triple nucleotide bases carried in transfer RNA that bring the correct amino acid in line with a messenger RNA during protein synthesis in the ribosome.

Gene A segment of DNA carrying specific codons for a particular polypeptide is called a gene. A DNA molecule consists of a large number of genes, each of which contains hundreds of thousands of nucleotides. The bacterial chromosome consists of a double-stranded molecule of DNA arranged in a circular form. When straightened, it is about 1,000 μm in length. The length of DNA is usually expressed as kilobases (1 kb = 1,000 base pairs). Bacterial DNA is about 4,000 kb while the human genome is about 3 million kb long.

Exons and introns Stretches of coded genes are called exons; non-coding sequences, which do not have any function are called introns.

Extrachromosomal genetic elements In addition to chromosomal DNA,

most bacteria possess extrachromosomal genetic elements. These are not essential for the normal life and functioning of the host bacterium but may confer properties such as drug resistance and toxigenicity, providing the bacteria with tools for survival.

Plasmids These are extrachromosomal circular DNA molecules present in the cytoplasm of bacteria and yeasts. They are capable of autonomous replication (independent replicons) and can be transferred from one cell to another. Plasmids have become important tools as vectors in genetic engineering. Plasmid DNA may sometimes be integrated with chromosomal DNA (episomes).

There are 2 types of plasmids:

- **Conjugative** plasmids, which are self-transmissible
- **Non-conjugative** plasmids, which are non-transmissible

Phenotype The **phenotype** (*phaeno* meaning display) is the **physical expression of the genotype in a given environment.** A cell may exhibit different phenotypic appearances in different situations; for example, *S. typhi* is normally flagellated but when grown in agar containing phenol, their flagella are not synthesised. This is only a phenotypic variation determined by the environment. It can be reversed when grown once again in broth media without phenol.

Genotype **All the genes present in a cell** (the genome) determine its **genotype**. This is the hereditary constitution of the cell, and is transmitted to its progeny. The genotype includes the complete genetic potential of the cell, all of which may or may not be expressed in a given environmental situation.

Genetic variations Genotypic variations occur due to alterations in the genome and are stable and heritable. They may occur by mutation or by one of the mechanisms of genetic transfer or exchange, which include transformation, transduction, lysogenic conversion and conjugation.

MUTATION

Mutations in genes are important in evolution. A mutation is a heritable change in DNA sequence caused by an alteration in the nucleotide

sequence. Mutation can be beneficial or harmful to the cell. It may be spontaneous due to processes within the cell or induced by an extraneous substance such as a mutagen in the environment.
The changes may be due to:

- Substitution of a nucleotide
- Deletion of a nucleotide
- Addition of nucleotides

Several methods have been developed for the detection of mutants. Of these, molecular methods are the preferred techniques because of their high sensitivity and specificity, and also because they are more time-saving than the classical methods like replica plating.

Details of the various types of mutations are presented in Table 5.1.

Table 5.1 Types of mutations in bacteria

S.No.	Type	Description
1	Missense mutation	Mutation where the triplet code is altered so as to specify an amino acid different from that normally located at a particular position in the protein.
2	Nonsense mutation	Deletion of a nucleotide within a gene may cause premature polypeptide chain termination by generating a nonsense codon.
3	Transversion	Substitution of a purine for a pyrimidine and vice versa in base-pairing is called transversion.
4	Suppressor mutation	Reversal of a mutant phenotype by another mutation at a position on the DNA distinct rom that of the original mutation.
5	Lethal mutation	Some mutations affect vital functions, and such mutants are non-viable. A type of lethal mutation which is of great interes is the ‘conditional mutation’.
6	Conditional lethal mutant	This type of mutation confers on the organism the ability to be able to live under certain conditions (permissive conditions). The commonest type of conditional mutant is the temperature-sensitive (ts) mutant,

		which allows the organism to live at the permissive temperature (say, 35°C), but not at the restrictive temperature (say, 39°C).
7	Frame-shift mutation	This is caused by the deletion or insertion of multiple nucleotides.
8	Multiple mutations	This is caused by extensive chromosomal re-arrangement.
9	Point mutation	Deletion or insertion of one or more bases is referred to as a point mutation.

Mutation in the gene may occur due to several changes in the base pair. They could be in the form of addition or deletion of a base or duplication, inversion or substitution as shown in Fig. 5.3.

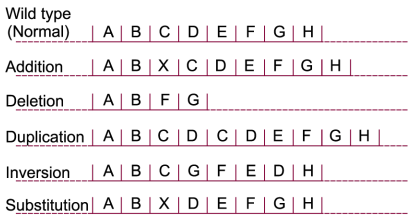

Wild type (Normal) | A | B | C | D | E | F | G | H |
Addition | A | B | X | C | D | E | F | G | H |
Deletion | A | B | F | G |
Duplication | A | B | C | D | C | D | E | F | G | H |
Inversion | A | B | C | G | F | E | D | H |
Substitution | A | B | X | D | E | F | G | H |

Fig. 5.3 Examples of types of mutations

- **Spontaneous mutations** Each gene undergoes mutation with a fixed frequency. Mutation rates of individual genes in bacteria range from 10^{-4} to 10^{-10} per bacterium per division.
- **Induced mutation** Though mutations occur spontaneously, their frequency can be increased by several agents (**mutagens**) such as UV rays, alkylating agents, acridine dyes, 5-bromouracil and 2-aminopurine.

Clinical applications of mutation

Mutation is of vital importance when it confers a survival advantage. If a streptomycin-resistant mutant of the tubercle bacillus develops in a patient under treatment with the drug, the resistant strain of the organism multiplies selectively and ultimately replaces the original

drug-sensitive population of bacteria. On the other hand, in a patient who is not on treatment, the mutation confers no survival advantage and, therefore, the preferential multiplication of the mutant does not occur. Mutations in bacteria may modify any characteristic of the bacterium. Mutants may vary in properties such as nutritional requirements, biochemical reaction, antigenic structure, morphological features, colony form, drug susceptibility, virulence and host range.

The practical importance of bacterial mutation lies mainly in the field of drug resistance and the development of live vaccines.

Detection of Mutation

i) Molecular techniques like **gene probes** and **sequencing** are used widely to detect changes in the bacteria due to mutation.

ii) '**Replica plating**' technique is used in the isolation of new resistant mutants in pure culture without prior exposure to the agent responsible for mutation (Fig. 5.4). It is rarely used in clinical laboratories.

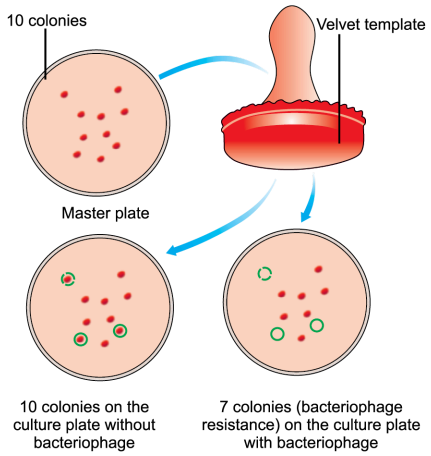

10 colonies
Velvet template
Master plate
10 colonies on the culture plate without bacteriophage
7 colonies (bacteriophage resistance) on the culture plate with bacteriophage

Fig. 5.4 Replica plating method to demonstrate mutation in a population of bacteria

GENE TRANSFER

Genetic information can be transferred from one bacterium to another by several methods.

Horizontal transfer occurs through transformation, transduction and

conjugation.

Vertical transmission occurs from the bacterium to its progeny via replication and recombination.

Horizontal Gene Transfer

1. Transformation

Transformation is the transfer of genetic information through the free DNA (Fig. 5.5). It was discovered in 1928 by **Griffith** who demonstrated that the injection of a mixture of live, non-capsulated (Type II) *S. pneumoniae* and heat-killed, capsulated (Type I) *S. pneumoniae* (neither of which were individually fatal) could kill mice. Subsequently, the pneumococcus which was isolated from the dead mice was found to be capsulated Type I [the gene (DNA) coding for the capsule had been transferred from the killed bacterium to the live, previously non-capsulated strain]. The nature of the transforming principle was identified as DNA by Avery, MacLeod and McCarthy in 1944.

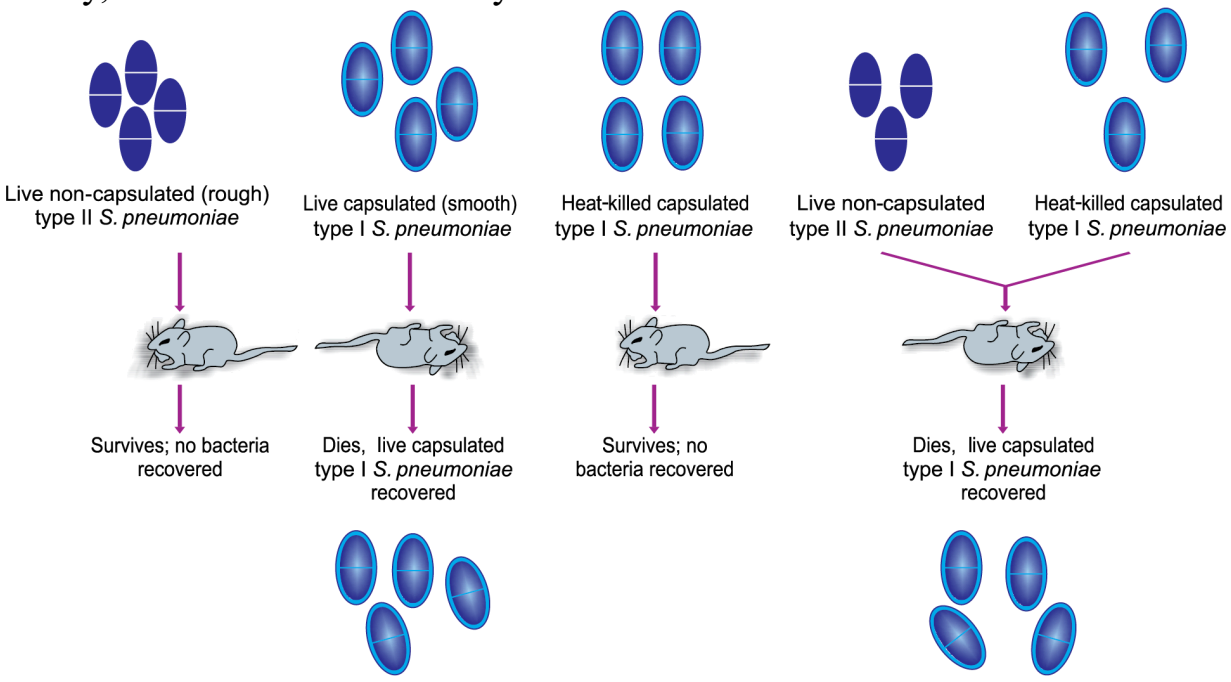

Live non-capsulated (rough) type II S. pneumoniae
Live capsulated (smooth) type I S. pneumoniae
Heat-killed capsulated type I S. pneumoniae
Live non-capsulated type II S. pneumoniae
Heat-killed capsulated type I S. pneumoniae
Survives; no bacteria recovered
Dies, live capsulated type I S. pneumoniae recovered
Survives; no bacteria recovered
Dies, live capsulated type I S. pneumoniae recovered

Fig. 5.5 Transformation experiment of Griffith

2. Transduction

The transfer of a portion of DNA from one bacterium to another by a

bacteriophage is known as transduction (Fig. 5.6). Bacteriophages are viruses that infect bacteria (details in Chapter 42).They consist of a nucleic acid core and a protein coat. During the assembly of bacteriophage progeny inside the infected bacteria, 'packaging errors' may occur. Besides its own nucleic acid, a segment of the host DNA may get incorporated into the viral genome. When the same bacteriophage infects another bacterium, the DNA taken up from the previous host is transferred to the recipient cell, which acquires new characteristics coded by the donor DNA.

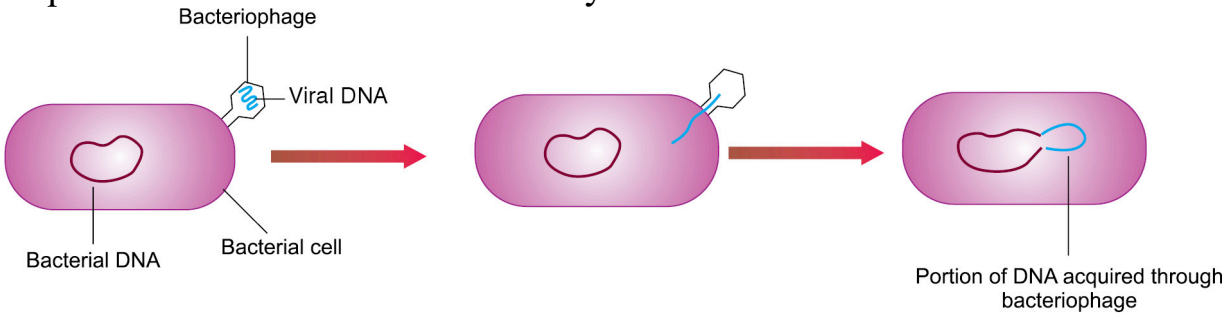


Fig. 5.6 Transduction

Types

Transduction may be:

- **Generalised**, when it involves any segment of the donor DNA at random.
- **Restricted**, when a specific bacteriophage transduces only a particular genetic trait. Restricted transduction has been studied in the 'lambda' phage of *E. coli*. The prophage lambda is inserted in the bacterial chromosome only between the genes determining galactose utilisation (*gal*) and biotin synthesis (*bio*) and, therefore, it transduces only either of these (Fig. 5.7).

Transduction is not confined to the transfer of chromosomal DNA. Episome and plasmid genes may also be transduced.

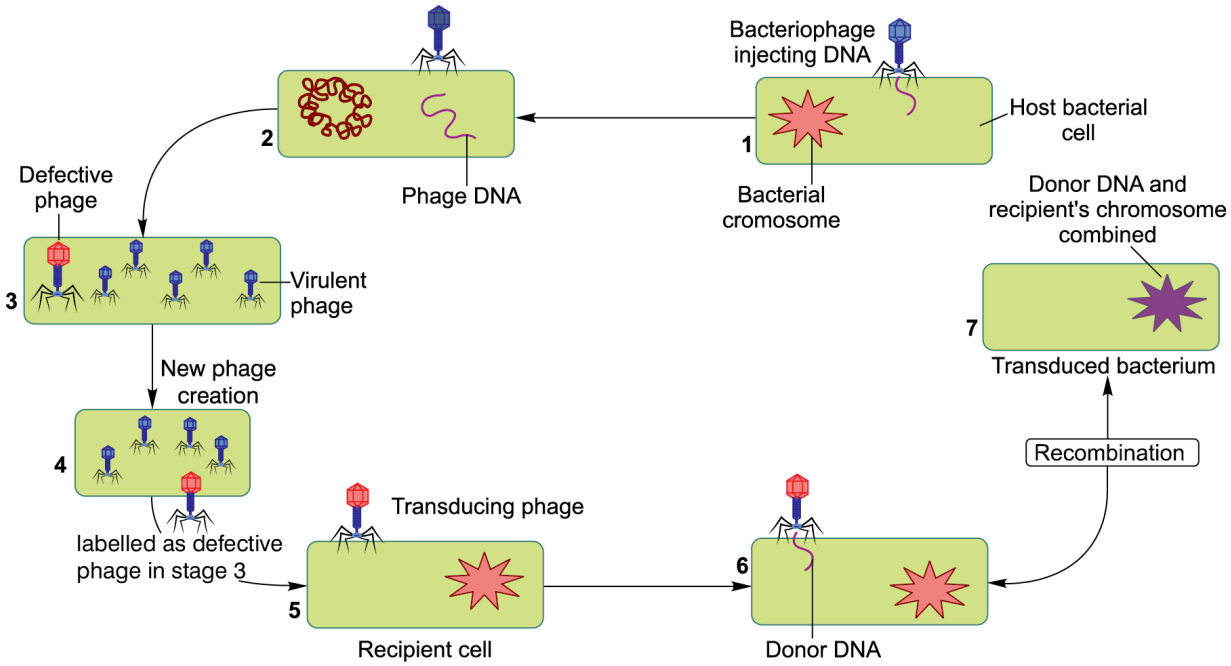


Fig. 5.7 Transduction via a bacteriophage from one bacterium to a second bacterium

3. Conjugation

Conjugation is a process whereby a donor ('male') bacterium conjugates or makes physical contact through a sex pilus with a recipient ('female') bacterium and transfers genetic elements into it (Fig. 5.8). Plasmids are the extrachromosomal elements that are frequently transferred by conjugation. Bacterial conjugation was first described by Lederberg and Tatum (1946) in a strain of *E. coli* called K_{12} and has been most extensively studied in this strain.

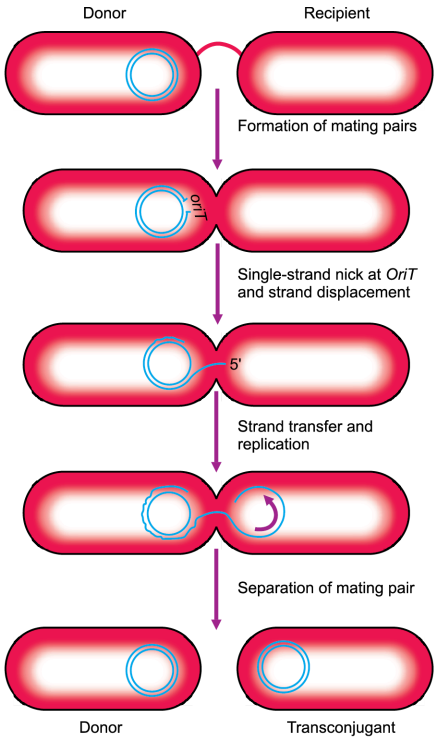


Fig. 5.8 Mechanism of DNA transfer through conjugation

The donor status of a cell is determined by the presence of a plasmid that codes for a specialised fimbria (**sex pilus**) that projects from the surface of the cell. The plasmid DNA replicates, and a copy of it passes from the donor to the recipient cell through the sex pilus (**conjugation tube**). As a result, the recipient attains donor status and can, in turn, conjugate with other recipient cells. The ability to conjugate is a transmissible or 'infectious' characteristic, which is located in a plasmid. Along with plasmid DNA, portions of the host DNA may also sometimes be transferred to the recipient. The donor DNA then combines with the DNA of the recipient, effecting genetic recombination. The plasmid responsible for this transfer is the '**fertility (F) factor**' and the bacteria possessing this trait are called F^+ bacteria. When other similar plasmids were discovered, the term **'transfer factor'** came to be used for all such plasmids that conferred on their host cells the ability to act as donors in conjugation.

The F factor

The F factor (originally called fertility factor) is a **transfer factor** that contains the genetic information necessary for the synthesis of the sex pilus and for self-transfer. It is devoid of other identifiable genetic markers such as

drug resistance. Cells carrying the F factor **(F^+ cells)** have no distinguishing features other than their ability to mate with F^- cells and render them F^+. The F factor is an episome that exists in some cells in the '**integrated state**' or is inserted into the host chromosome. Such cells can transfer chromosomal genes to recipient cells with **high frequency** and are known as **Hfr cells**. Following conjugation with an Hfr cell, an F^- only rarely becomes F^+, though it receives chromosomal genes from the donor.

F prime (F') factor

Conversion of an F^+ cell into the Hfr state is reversible. When the F factor reverts from the integrated state to the free state, it may sometimes carry with it some chromosomal genes from near its site of attachment. Such an F factor, in which certain chromosomal genes are incorporated, is called an F prime (F') factor.

Sexduction When an F' cell mates with a recipient, it transfers, along with the F factor, the host genes incorporated with it. This process of transfer of host genes through the F' factor resembles transduction and has, therefore, been called sexduction (Fig. 5.9).

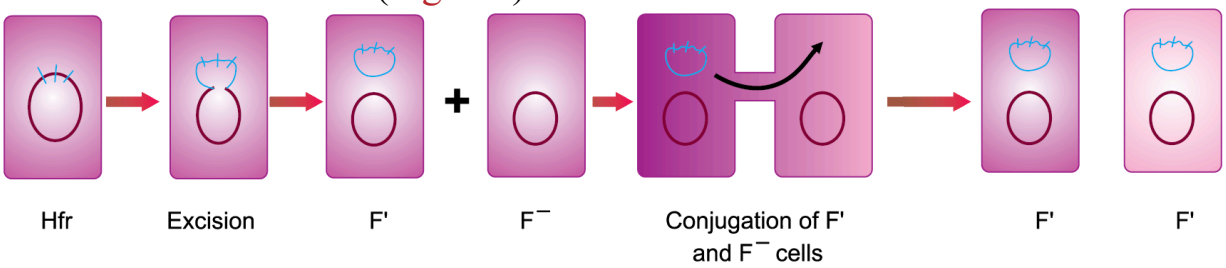


Fig. 5.9 Sexduction—the integrated F factor of an Hfr cell may revert to the cytoplasmic state; during excision, some host genes may be incorporated in the F' factor (F); when an F' cell mates with an F^- cell, the host gene is transferred to the recipient

Colicinogenic (Col) factor

Several strains of coliform bacteria produce **colicins**—antibiotic-like substances that are specifically and selectively lethal to other enterobacteria. As similar substances are produced by bacteria other than coliforms (pyocin by *Pseudomonas pyocyanea*, diphthericin by *Corynebacterium diphtheriae*), the name **bacteriocin** has been given to this group of substances. The specificity of the action of bacteriocins enables intraspecies classification of certain bacteria (for example, *Shigella sonnei* and *P. aeruginosa*).

Colicin production is determined by a plasmid called the **Col factor**, which resembles the F factor in promoting conjugation, leading to self-transfer and, at times, the transfer of chromosomal segments.

Resistance transfer factor (RTF)

This plasmid is of great medical importance as it leads to the spread of multiple drug resistance among bacteria.

This extrachromosomal mechanism of drug resistance was first reported by Japanese workers (1959) investigating the sudden increase in infections caused by *Shigella* strains resistant simultaneously to sulphonamides, streptomycin, chloramphenicol and tetracycline. They observed that patients excreting such *Shigella* strains also shed in their feces *E. coli* strains resistant to the same drugs. **Transfer of multiple drug resistance was demonstrated between *E. coli* and *Shigella* strains both in vitro and in vivo**. The resistance is plasmid-mediated and is transferred by conjugation. This mechanism of drug resistance is known as **transferable, episomal** or **infectious drug resistance**.

Components of RTF

This plasmid consists of two components (Fig. 5.10):

a) The transfer factor called the **resistance transfer factor (RTF),** which is responsible for conjugational transfer.

b) **Resistance determinant (*r*)** for each of the several drugs. The whole plasmid (RTF + *r* determinants) is known as the **R factor**. An R factor can have several *r* determinants, and resistance to as many as eight or more drugs can be transferred simultaneously.

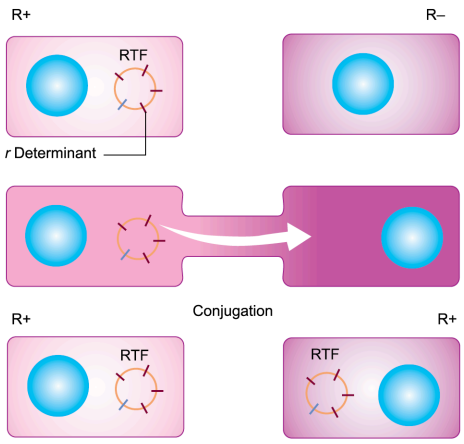


Fig. 5.10 Transferable drug resistance; the R^+ cell carries the R factor, consisting of the RTF and *r* determinants; its transfer to a sensitive R^- bacterium converts the recipients into resistant R^+ cells

Sometimes, the RTF may dissociate from the *r* determinants, the two components existing as separate plasmids. In such cases, though the host

cell remains drug-resistant, the **resistance is not transferable**. The RTF can have determinants other than those for drug resistance attached to it. **Enterotoxin and hemolysin production in some enteropathogenic *E. coli* are transmitted by this transfer factor**.

Clinical implications of transferable drug resistance

Transferable drug resistance is seen widely in various pathogenic and commensal bacteria of humans and animals, such as Enterobacteriaceae, *Vibrio, Pseudomonas* and *Pasteurella*. The transfer can be effected readily in vitro but in the normal gut, it is inhibited by several factors such as anaerobic conditions, bile salts, alkaline pH and the abundance of anaerobic gram-positive bacteria, minimising the chances of contact between donor cells and suitable recipient cells. On the other hand, in the intestines of persons on oral antibiotic therapy, transfer occurs readily due to the destruction of the sensitive normal flora and the selection pressure produced by the drug.

Vertical Gene Transmission

During replication of a bacterium, its genetic information is passed on to its progeny. The parent DNA replicates into two double-stranded DNA, each containing a template strand and a newly synthesised strand. Small pieces of chromosomes are replicated in this way, and move to one end of the bacterial cell. They are enclosed in plasma membrane and a new cell wall develops, giving rise to daughter cells containing the genes.

Bacterial Recombination

This is a technique whereby a piece of DNA (containing specific genes) that has been created artificially from two or more sources is incorporated into a single bacterial molecule. This results in the expression of the particular genetic information by the bacteria and its progeny. This helps in generating large quantities of DNA carrying the specified genetic information. The different types of recombination are:

i) **Homologous recombination**—occurs between DNA molecules that have very similar sequences.

ii) **Non-homologous recombination**—occurs between pieces of DNA in which there is no large similarity in sequences.

iii) **Site-specific recombination**—occurs between short sequences present in dissimilar molecules.

iv) **Replicative recombination**—generates a new copy of a segment of the DNA.

GENETIC MECHANISMS OF DRUG RESISTANCE IN BACTERIA

Bacteria may acquire drug resistance by:

1. **Mutation**
2. By one of the methods of **genetic transfer** (Table 5.2)
3. **Physical mechanisms** of resistance, which include:

- Decreased permeability to the drug
- Development of alternative metabolic pathways
- Production of enzymes that inactivate the drugs

Table 5.2 Comparison of mutational and transferable drug resistance

Mutational drug resistance	Transferable resistance
Bacteria are resistant to one drug at a time.	Bacteria are resistant to multiple drugs.
Low-degree resistance is seen.	High-degree resistance is seen.
It can be overcome by high drug dose.	High doses are ineffective.
Drug combinations cannot prevent development of resistance.	Drug combinations can prevent development of resistance.
Resistance does not spread.	Resistance spreads to same or different species.
Mutants may be defective.	Mutants are not defective.

Mutational Resistance

This is mainly of two types:

1. **Stepwise mutation**, as seen with **penicillin**, where high levels of resistance are achieved only by a series of small-step mutations.
2. '**One-step' mutation**, as seen with **streptomycin**, where the mutants differ widely in the degree of resistance, some exhibiting low resistance, while others may be highly resistant, some even being streptomycin-dependent.

Clinical implications of mutational resistance

In clinical practice, mutational resistance is of great importance in **tuberculosis.** If a patient is treated with streptomycin alone, the bacilli initially die in large numbers but soon, resistant mutants appear and multiply unchecked. If two or more anti-tuberculous drugs are combined, re-population by resistant mutants does not occur. This is because a mutant resistant to one drug will be destroyed by the other drug. This is the rationale behind combined treatment in tuberculosis. Mutants exhibiting resistance to multiple drugs simultaneously are rare. However, in spite of this knowledge, inadequate or inappropriate treatment over the years has caused extensive resistance in tubercle bacilli, leading to a pandemic of multidrug-resistant tuberculosis (**MDR-TB**) across the world.

Acquisition of resistance by transduction is common in staphylococci. The penicillinase plasmids, which are transmitted by transduction, may also carry determinants for resistance to mercuric chloride and erythromycin.

Genetic Transfer

Plasmid-mediated transferable drug resistance

This is mediated by the **R factor**. It is the most important method of drug resistance. Some characteristics of plasmid-mediated antibiotic resistance are as follows:

- Acquisition of an R factor **simultaneously confers resistance to several drugs** and, therefore, treatment with a combination of drugs is not useful.
- The resistance is due to the production of **degrading enzymes**, and the level of resistance is usually high.
- Resistance may be **transferred between bacteria** of different taxonomic groups.
- Resistant mutants usually have a **lower growth rate and reduced virulence** as compared to the wild strains.
- Bacteria carrying R factors are apparently normal in other respects.
- R factors in some cases may lead to enhanced virulence.
- Multiple drug resistance was initially seen in bacteria causing diarrhea and other mild infections that did not call for antibiotic treatment as a routine.

- Subsequently, it spread to virtually all pathogenic bacteria affecting humans and animals, making antibiotic therapy of infections ineffective.
- Transferable drug resistance is now **universal in distribution** and involves all antibiotics in common use.
- Its incidence is **directly proportional to the frequency of use of antibiotics** in the area.
- Bacteria carrying R factors can be transmitted from animals to humans. Hence, the indiscriminate use of antibiotics in veterinary practice or in animal feeds can also lead to an increase in multiple drug resistance in the community.
- The addition of antibiotics in animal feeds has, for this reason, been prohibited by legislation in some countries.
- Widespread resistance has considerably diminished the clinical efficacy of most antibiotics.
- In the laboratory, R factors may sometimes be eliminated by treating bacteria with acridine dyes or ethidium bromide.
- In the community, the only way to prevent widespread dissemination of multiple resistance is to restrict the use of antibiotics to the essential minimum.

Transposable genetic elements

Certain structurally and genetically discrete segments of DNA have been identified that have the ability to move around in a 'cut-and-paste' manner between chromosomal and extrachromosomal DNA molecules within cells. These DNA molecules are called **transposons** ('**jumping genes**') and this mode of genetic transfer, **transposition** (discovered in plants by Barbara McClintock, for which she was awarded the Nobel Prize for Medicine in 1983). A transposon is a segment of DNA with one or more genes in the centre, and the two ends carrying **'inverted repeat' sequences** of nucleotides (Fig. 5.11). Small transposons (1–2 kb) are known as '**insertion sequences**' or **IS.** Transposons attach at certain regions of chromosomal, plasmid or phage DNA. Insertion of a transposon leads to the acquisition of new characteristics by the recipient DNA molecule. Unlike plasmids, transposons are not self-replicating and depend on chromosomal or plasmid

DNA for replication.

Characteristics transferred by transposons may sometimes confer survival advantages under appropriate environmental conditions. It has been suggested that the resistance-determinant segments of the R factors may have evolved as collections of transposons, each carrying a gene that confers resistance to one or several antibiotics.

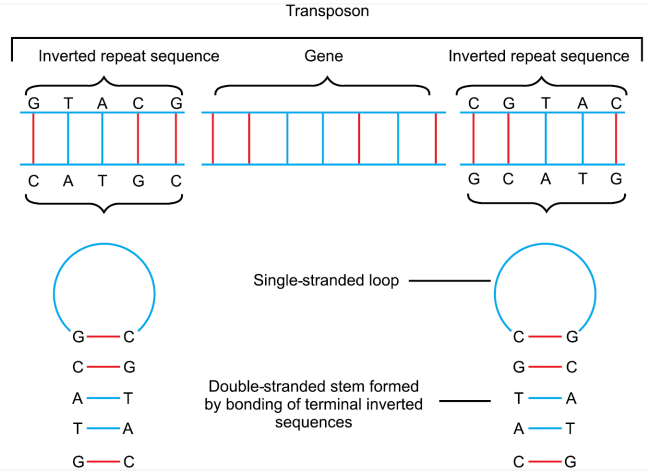


Fig. 5.11 Structure of transposon

Key points

- Deoxyribonucleic acid (DNA) carries genetic information, which is transcribed onto ribonucleic acid (RNA) and then translated into the particular polypeptide (DNA → RNA → polypeptide).
- Plasmids are circular DNA molecules present in the cytoplasm of bacteria, capable of autonomous replication (independent replicons).
- The total genetic constitution of an organism is its genotype. The genotype of a cell is determined by the genetic information contained in its entire DNA content or genome.
- The characteristics displayed by an organism in any given environment comprise its phenotype.
- Mutation is a random, undirected, heritable variation caused by an alteration in the nucleotide sequence at some point of the DNA of the cell.
- Each gene undergoes spontaneous mutation with a fixed frequency. Mutation rates of individual genes in bacteria range from 10^{-4} to 10^{-10} per bacterium per division.
- Several agents (mutagens) such as UV rays, alkylating agents, acridine dyes, 5-bromouracil and 2-aminopurine can induce mutations.
- Three important modes of genetic transfer are:
  i) Transformation, where there is direct uptake of donor DNA by the recipient cell, which may be natural or forced.

ii) Transduction, where donor DNA is carried in a bacteriophage and is transferred to the recipient by the mechanism used for phage infection.

iii) Conjugation, where the donor cell contributes energy and building blocks to the synthesis of a new DNA strand, which is physically transferred into the recipient cell, usually by a tube called a sex pilus.

- Transferable drug resistance mediated by the R factor is the most important mode of drug resistance.
- Certain structurally and genetically discrete segments of DNA have the ability to move around in-between chromosomal and extrachromosomal DNA molecules within cells. These DNA molecules are called transposons ('jumping genes') and this mode of genetic transfer, transposition.

Questions

Essays

1. Enumerate the methods of transfer of genetic materials in bacteria. Explain the mechanism of any one.
2. Describe the differences between resistance acquired by mutation and transferable drug resistance.

Short Answers

1. Transformation
2. Transduction
3. Conjugation
4. Plasmids
5. Transferable drug resistance
6. Mutations in bacteria
7. Transposons

CHAPTER 6

Molecular Techniques Applied to Microbiology

Chapter navigation

- Molecular methods applied to disease detection and diagnosis
  - Amplification techniques
  - Non-amplification techniques
  - DNA sequencing
- Genetic engineering

Discoveries in microbial genetics have provided the basis for the analysis and manipulation of DNA using molecular techniques. Some of the techniques in molecular genetics and their applications in the field of epidemiology, in disease detection and in the rapid diagnosis of infectious diseases are discussed in this chapter.

MOLECULAR METHODS APPLIED TO DISEASE DETECTION AND DIAGNOSIS

The basis of DNA manipulation is the use of restriction endonucleases (restriction enzymes), which are microbial enzymes that cleave double-stranded DNA at specific oligonucleotide sequences. Many such enzymes which act at different nucleotide sequences (for example, *Eco* RI, *Hind* III and *Taq* I) have been recognised. Restriction enzymes split DNA strands into fragments of varying lengths, which can be separated by gel electrophoresis.

Methods for Detecting Microorganisms

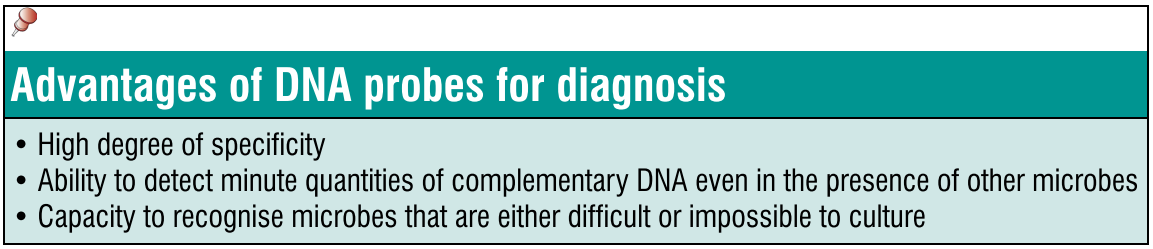
Several molecular techniques have been used to detect microbial agents in

clinical samples. Some of the commonly used methods are described below.

Nucleic Acid Probes

DNA probes

DNA strands can be detected using specific DNA probes, which hybridise with the DNA during DNA or RNA synthesis. These probes are otherwise known as nucleic acid probes. The probes are radioactively labelled, biotinylated copies of cloned single-stranded DNA fragments, usually 20–25 nucleotides long and containing unique nucleotide sequences which can be used for the detection of homologous DNA by hybridisation. DNA probes are used in the diagnosis of infectious diseases. Probes containing sequences unique to the microbe (strain, species or group) to be detected can be added to microbial cultures, body fluids, tissues or other clinical materials suspected to contain the microbe or its DNA. The DNA probe hybridises with the complementary specific sequences on the microbe's DNA.



Advantages of DNA probes for diagnosis

- High degree of specificity
- Ability to detect minute quantities of complementary DNA even in the presence of other microbes
- Capacity to recognise microbes that are either difficult or impossible to culture

DNA probes for the detection of many pathogens are now commercially available.

Blotting Techniques

These are the techniques used for detection of DNA, RNA or proteins of interest.

Southern blotting

The technique for **identifying DNA fragments by DNA–DNA hybridisation** is called Southern blotting, after E. M. Southern who devised it. DNA fragments obtained by restriction enzyme digestion and separation on gel can be transferred from the gel by blotting them onto nitrocellulose

or nylon membranes that bind to the DNA. The DNA bound to the membrane is denatured (converted to the single-stranded form) and treated with radioactive single-stranded DNA probes. These probes hybridise with homologous DNA to form radioactive double-stranded segments, which can be detected on an X-ray film. This technique is used to detect genetic disorders, cancers such as monoclonal leukaemia, etc.

Northern blotting

An analogous procedure for the analysis of RNA has been called Northern blotting (as opposed to Southern blotting). Here, the **RNA mixture is separated by gel electrophoresis, blotted and identified** using labelled DNA or RNA probes. For example, it is used to detect mRNA in a sample for gene expression.

Western blotting

A similar technique for the identification of proteins (antigens) is called **immunoblotting** (or, in conformity with other blotting techniques, western blotting). Here, the **protein antigen mixture is separated by SDS–PAGE** (sodium dodecylsulphate–polyacrylamide gel electrophoresis), **blotted on to nitrocellulose strips and identified** by radiolabelled or enzyme-labelled antibodies as probes. The western blot test is used as a confirmatory test for the diagnosis of HIV antibody in sera; the TORCH panel (Toxoplasma, Rubella, Cytomegalo and Herpes antibodies) is used to screen antenatal women with bad obstetric history for intrauterine infection and several other infectious diseases.

Eastern blotting

The **eastern blot** is a biochemical technique used to analyse protein post-translational modifications (PTM) including the addition of lipids, phosphates and glyco-conjugates. It is most often used to detect carbohydrate epitopes. Thus, eastern blotting can be considered an extension of the biochemical technique of western blotting. Multiple techniques have been described by the term eastern blotting, most of which use proteins blotted from SDS-PAGE gel on to a PVDF or nitrocellulose membrane. Transferred proteins are analysed for post-translational modifications using probes that may detect lipids, carbohydrate, phosphorylation or any other protein

modification. The term ‘eastern blotting’ should be used to refer to methods that detect targets through specific interaction of the PTM and the probe, distinguishing them from a standard far-western blot. In principle, eastern blotting is similar to lectin blotting (i.e., detection of carbohydrate epitopes on proteins or lipids).

DNA Amplification Techniques

Polymerase chain reaction (PCR)

Amplification of specific DNA sequences (or genes) was invented by Kary B. Mullis in 1983, for which he won the Nobel Prize in Chemistry in 1993. It is a chain reaction in which a small fragment of the DNA of interest serves as the template for producing the primers that initiate the reaction. One DNA molecule is used to produce two copies, which then form the template for the next reaction in a thermocycler. Thus, it is possible to obtain several copies of the DNA of interest, and thereby, an abundant quantity of specific DNA from a minuscule source (Fig. 6.1).

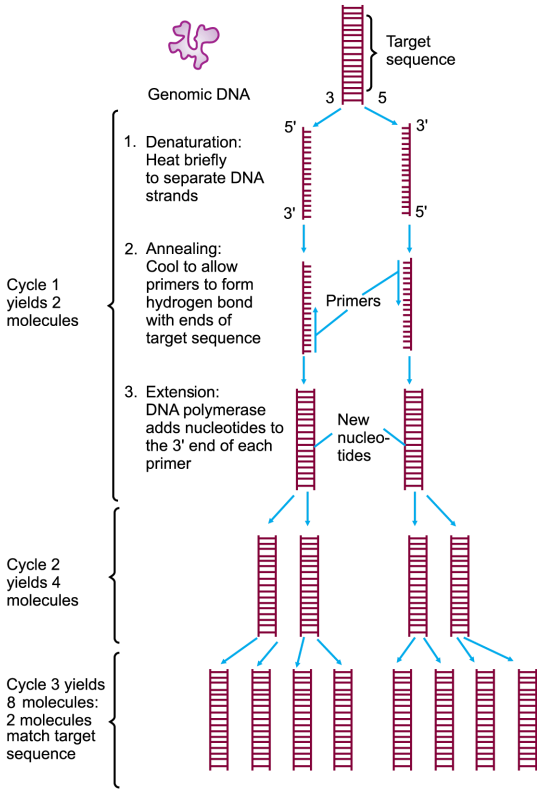

Target sequence
Genomic DNA
3
5
5'
3'
3'
5'
1. Denaturation: Heat briefly to separate DNA strands
2. Annealing: Cool to allow primers to form hydrogen bond with ends of target sequence
Primers
3. Extension: DNA polymerase adds nucleotides to the 3' end of each primer
New nucleo-tides
Cycle 1 yields 2 molecules
Cycle 2 yields 4 molecules
Cycle 3 yields 8 molecules: 2 molecules match target sequence

Fig. 6.1 Diagrammatic representation of amplification of genomic DNA by PCR

Applications of PCR

With its enormous capacity to amplify DNA, PCR (Fig. 6.2) is a versatile tool, useful in diverse areas such as:

- Diagnosis of infectious diseases
- Genetics of neoplastic diseases
- Forensic investigations
- Archaeo-biological studies of ancient specimens
- Examination of phylogenetic relationships in evolution

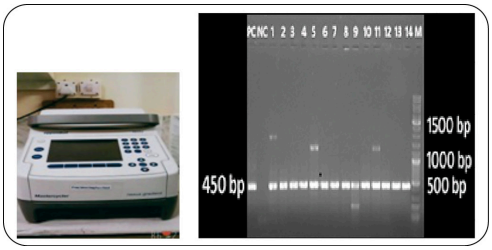


Fig. 6.2 (a) Thermocycler (conventional PCR) to amplify the desired DNA and (b) gel documentation of amplified DNA bands of specific base pairs compared to a molecular weight ladder (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Based on the principle of PCR, other target amplification systems have been developed.

Reverse transcriptase PCR (RT-PCR) The conventional PCR technique can only amplify the dsDNA (double-stranded DNA) sequences. Therefore, RNA viruses cannot be amplified by this method directly. In this modification (RT-PCR), with the help of reverse transcriptase (RT) enzyme, a complementary copy of DNA is made from RNA and this cDNA is then used as a template for PCR.

Quantitative PCR (qPCR) This is real-time PCR (earlier also called RT-PCR with which it could be confused). The advantage that this method has over the conventional method is that it quantifies the PCR by monitoring the amplification process while the PCR is ongoing, i.e., in real time (Fig. 6.3). Hence it is called real-time PCR. This method gives the estimate of the pathogen DNA load in the test specimen. Samples can be processed for rapid detection of specific DNA load, e.g., hepatitis B DNA viral load. The detection is done using:

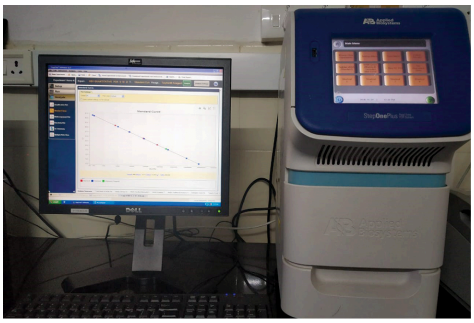


Fig. 6.3 Quantitative PCR (qPCR); it is a real-time PCR which quantifies the PCR in real-time (*Source:* Pushpagiri Institute of Medical Sciences and Research Centre, Thiruvalla, Kerala)

- **Non-specific fluorescent dyes** like SYBR green that intercalate with dsDNA as it gets synthesised.
- **Specific set of DNA probes** which are labelled with fluorescent reporters that get detected only after the probe hybridises with the complementary DNA sequence. The fluorescent reporter signal strength is directly proportional to the number of amplified DNA molecules.

Nested PCR In conventional PCR, sometimes, the primers bind to non-specific regions of DNA. In order to take care of this non-specificity, a second set of specific primers is used in the successive cycles to amplify a secondary target within the first target.

Multiplex PCR Primers specific to more than one pathogen are used simultaneously in the same reaction to give multiple PCR products.

Non-amplification Tests

Amplification requires the use of a thermocycler, temperature regulation and several cyclical runs. To circumvent these, non-amplification techniques have been developed.

Loop-mediated isothermal assay (LAMP)

This is a simple technique, perfomed at constant temperature. There is no need to carry out the reaction in a series of different temperatures and, therefore, there is no need for a thermocycler.

This method has potential for use as a screening test in the field or as a point-of-care test. In the common format, four sets of primers are used to

bind to six regions on the target DNA. Primers with a specific nature, called '**loop primers**', help in the yield of higher amounts of DNA. The polymerase enzyme used in this method is also special because it has a 'high strand displacement' activity.

DNA microarray

It is a method in molecular biology in which many DNA spots are coated on a solid surface, e.g., silicon or glass chip. These spots are specific DNA sequences that serve as probes. These probes hybridise with cDNA in the test sample, where the DNA in the sample does not have to be amplified. This method can detect a large number of nucleic acid sequences in the target simultaneously.

Molecular epidemiology Molecular techniques are used for the matching of microbial isolates for epidemiological purposes by:

- Plasmid profile analysis
- Genomic fingerprinting
- Typing of microorganisms

Sequencing-Based Assays

Genetic mapping

Complete genomic sequencing of bacteriophages, viruses, bacteria and their plasmids, and even of some eukaryotes, including mammals has been carried out. Sequencing techniques have enabled the study of the whole genome or pan-genome of bacteria for use in diagnosis and research. Their sucess led to the initiation of the **Human Genome Project**, the most expensive and ambitious scientific project thus far undertaken in biology. The results of this mammoth study became available at the beginning of the twenty-first century and opened vistas in human biology and medicine. At the same time, genome mapping has been riddled by controversies and dilemmas that go beyond medicine and into ethical issues.

DNA sequencing technology has advanced to high turnover and high-speed techniques, as compared to the conventional Sanger's sequencing method. Today, it is possible to sequence several DNA fragments at a time, followed by their bioinformatics analyses. These methods are presently

used for research purposes but also have a potential for rapid infectious disease diagnosis. The cost of equipment and infrastructure like computer capacity and storage, expertise in analysis of a big data, etc., are limitations for its wider use at present.

Advantages and disadvantages of molecular methods in disease diagnosis

Advantages
- Highly sensitive and specific.
- Can be applied directly to the specimen, even when only minute quantities are available.
- Relatively rapid compared to culture.
- Can detect the disease in the early stages.
- Can detect several characteristics of the microorganism, e.g., antibiotic resistance and virulence.
- Can be used for epidemiological purposes to trace the origin or source of infection.

Disadvantages
- Comes at a high cost (which has come down due to wider application).
- Requires specific technology and equipment.

APPLICATION OF GENETIC ENGINEERING IN MICROBIOLOGY

The most important application of molecular genetics in biotechnology is genetic engineering or **recombinant DNA (rDNA) technology**. This is a process by which the DNA of a microorganism can be manipulated. Its more common use involves **cloning**. The steps involved are: the isolation of the genes coding for any desired protein from microorganisms or from cells of higher forms of life including human beings and their introduction into suitable microorganisms, in which the genes would be functional, directing the production of the specific protein. Such cloning of genes in microorganisms enables the preparation of the desired protein in pure form, in large quantities and at a reasonable cost.

DNA is cleaved by specific enzymes called **restriction endonucleases** (as described earlier in the chapter), and the fragments containing the desired genes are isolated. These fragments are incorporated into suitable vectors or carriers, such as plasmids or temperate bacteriophages, for insertion into microorganisms. The microorganism commonly employed is ***E. coli*** $\mathbf{K_{12}}$,

though many other bacteria and yeasts have also been used. These bacteria multiply and produce a large amount of the desired gene product.

Commercial applications of genetic engineering

- Cloned human insulin, interferons, somatostatin, growth hormones and many other biologicals have already been marketed.
- Safer vaccines can be produced by cloning the protective antigens of pathogens, as has already been done in the case of foot and mouth disease, hepatitis B and rabies viruses.
- Application for antigen preparation in kits has also paved the way for various sensitive and specific tests for the diagnosis of infectious diseases.

Key points

- PCR and its modifications are based on the amplification of the small amount of DNA sequences in a test sample to numbers that can be detected.
- Molecular diagnosis of infectious diseases and understanding the biology of the infectious agents have been made possible by studying the genetic elements of the microorganisms.
- Genetic engineering or recombinant DNA (rDNA) technology involves the isolation of the genes coding for any desired protein from microorganisms or from cells of higher forms of life (including human beings), and their introduction into suitable microorganisms, in which the genes would be functional, directing the production of the specific protein.

Questions

Essay

Describe the molecular techniques used in clinical microbiology for the diagnosis of infectious diseases.

Part II: Immunology

Part II: Immunology

CHAPTER 7

Introduction to Infection and Immunity

Chapter navigation

- Infection
  - Source and transmission
  - Microbial virulence factors and their regulation
  - Pathogenesis
- Immunity
  - Innate immunity
  - Acquired immunity
  - Measurement of immune response

INTRODUCTION

Invasion and multiplication of microorganisms in a host constitutes an infection. It does not invariably result in disease. In fact, disease is a rare consequence of infection if the host's immunity is intact. The immune system in living organisms is responsible for protecting them from invading foreign substances including pathogens. It has evolved into a specialised system which regulates protection by several mechanisms. The immune system is highly adaptable, involving several different cells and molecules that help to specifically recognise foreign objects. The system has two important functions—first, to recognise a substance as foreign (to the host) and second, to respond to it. The following are the definitions of some basic terms to be familiar with before studying infections and immunity.

Parasites Microbes that can establish themselves and multiply in hosts are called parasites. An invading or foreign organism may be classified as either

a pathogen (from Greek pathos meaning ‘suffering’, and gen meaning ‘produce’, i.e., disease-producing) or a commensal (from Latin com meaning ‘sharing’, and mensa meaning ‘table’, i.e., ‘living together’).
Pathogens These are microorganisms that are capable of producing disease in the host.

Commensal Microbes that live in complete harmony with the host without causing any damage to it are called commensals. The normal bacterial flora of the body are largely commensals.

Many commensals behave as facultative pathogens, producing disease when the immunity of the host is lowered.

INFECTION

Infection occurs when an organism enters the body and establishes itself, resulting in a disease process in the host. The organism causing the disease is known as the **pathogen**, and the process by which it attacks the host is known as **pathogenesis**. A disease resulting from an infection is known as an **infectious disease.**

Infestation This refers mainly to ectoparasites like lice, fleas and ticks, which live parasitically on the host, producing harmful effects. It also refers to intestinal parasitic infestations by the helminths.

Colonisation This refers to the presence of organisms on the host, which do not cause infection but which have the potential to become pathogenic if the immunity of the host is compromised. For example, coagulase-negative staphylococcus is a coloniser of the skin but it can cause bloodstream infection in a patient on chemotherapy, who is on an intravenous catheter line.

Infections may be classified in various ways:

- ***Primary infection*** It is the initial infection in a host by a parasite.
- ***Re-infection*** Subsequent infections by the same parasite in the host are referred to as re-infection. For example, malaria can recur in an individual who is exposed to it repeatedly.
- ***Secondary infection*** When a new parasite causes an infection in a host whose resistance is lowered by a pre-existing infectious

disease, it is referred to as a secondary infection, e.g., fungal infection of the lung in a patient with pulmonary tuberculosis.

- ***Focal infection*** More appropriately called **focal sepsis**, this indicates a condition caused by infection or sepsis at localised sites, e.g., appendicitis or tonsillitis.
- ***Cross-infection*** When a patient already suffering from one infection or disease contracts a new infection which is transmitted from another host or another external source, it is called a cross-infection. Most hospital-acquired infections are due to cross-infection.

> Cross-infections occurring in hospitals are called **nosocomial infections** (from Greek *nosocomion* meaning 'hospital'). They are currently termed as **health care-associated infections (HAI)**.

- ***Iatrogenic infection*** These are infections resulting from investigative, therapeutic or other procedures which are physician-induced, e.g., abscesses resulting from injections.
- ***Endogenous or exogenous infection*** Depending on whether the source of the infection is from within or outside the host's own body, it is called either an endogenous or an exogenous infection, e.g., peritonitis following intestinal perforation (endogenous flora from the gut).

Based on the clinical effects, infections may be classified as follows:

- ***Inapparent infection (subclinical infection)*** It is an infection in which the clinical effects are not apparent.
- ***Atypical infection*** This refers to an infection in which the typical or characteristic clinical manifestations of the particular infectious disease are not present.
- ***Latent infection*** It is an infection in which some parasites, following infection, remain in the tissues in a latent or hidden form, proliferating and producing clinical disease when the host's resistance is lowered.

SOURCES OF INFECTION

The source of microorganisms that cause infections can be one of the following:

1. Humans
2. Animals
3. Insects
4. Environment

1. Humans

The commonest source of infections in humans are humans themselves. A parasite may originate from a patient or a carrier.

A **carrier** is a person who harbours the pathogenic microorganism without suffering any ill effect because of it. They may be one of the following types:

- **Healthy carrier**—one who harbours the pathogen but has not suffered from the disease caused by the pathogen.
- **Convalescent carrier**—one who has recovered from the disease and continues to harbour the pathogen in his body.

Depending on the duration of carriage, carriers are classified as temporary and chronic.

- **Temporary carriers**—convalescent carriers in whom the microorganisms persist for less than six months.
- **Chronic carrier**—a state that may last for several years after the infection and sometimes even for the rest of the person's life.
- **Contact carrier**—a person who acquires the pathogen from a patient.
- **Paradoxical carrier**—a carrier who acquires a pathogen from another carrier.

2. Animals

Many pathogens are able to infect both humans and animals (Fig. 7.1). Animals may, therefore, act as sources of human infection. In some instances, the infection in animals may be asymptomatic. Such animals

serve to maintain the parasite in nature and act as reservoirs of human infections. They are called **reservoir hosts**.

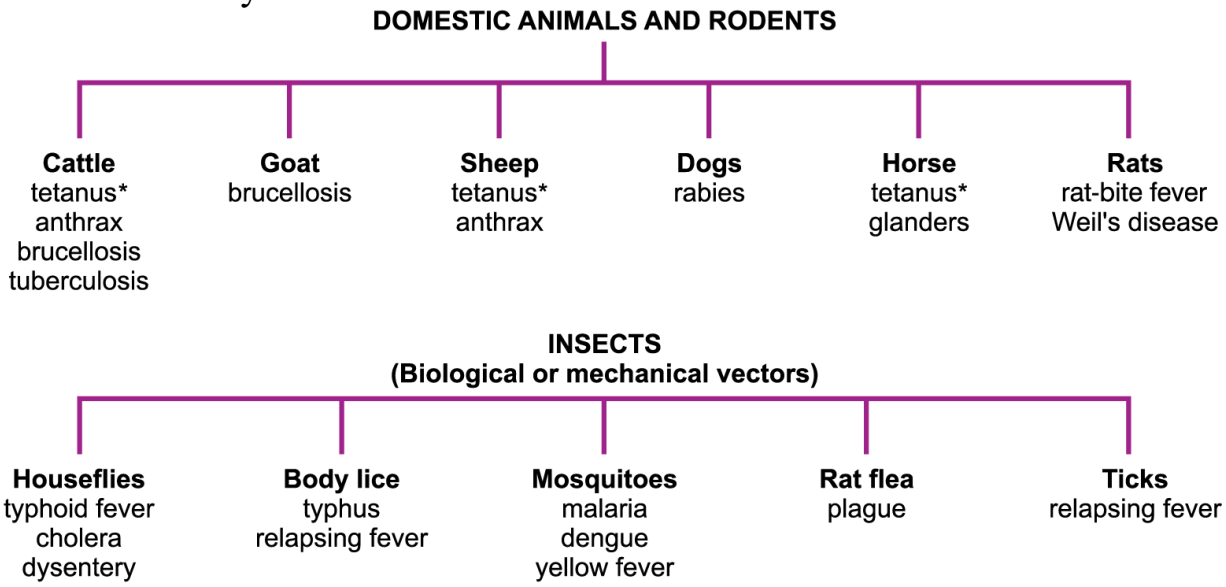


Fig. 7.1 Possible sources of infection (*Tetanus bacilli are present in the feces of these animals)

Zoonoses

Infectious diseases transmitted from animals to human beings are called zoonoses. Zoonotic diseases may be:

- bacterial (e.g., plague from rats)
- viral (e.g., rabies from dogs)
- protozoal (e.g., toxoplasmosis from cats)
- helminthic (e.g., hydatid disease from dogs)
- fungal (e.g., zoophilic dermatophytes from cats and dogs)

3. Insects

Bloodsucking insects may transmit pathogens to human beings. The diseases so caused are called **arthropod-borne diseases**. Insects such as mosquitoes, ticks, mites, flies, fleas and lice, which transmit infections are called **vectors.**

Transmission may be via:

- ***Mechanical vectors*** The pathogen is transmitted mechanically by the vector (e.g., transmission of dysentery or typhoid bacilli by

the domestic fly).

- ***Biological vectors*** The pathogen multiplies in the body of the vector, often undergoing part of its developmental cycle inside it (e.g., *Aedes aegypti* mosquito in dengue, *Anopheles* mosquito in malaria). Biological vectors transmit infections only after the pathogen has multiplied in them sufficiently or has undergone a developmental cycle.

Extrinsic incubation period refers to **the time period between the entry of the pathogen into a vector and the vector becoming infective**.

Infection is maintained in such insects by transovarial or transstadial passage (e.g., ticks in relapsing fever).

4. Environment

- ***Soil and water*** Some pathogens can survive in the soil for very long periods of time. Spores of the tetanus bacilli remain viable in the soil for several decades. Fungi (*Histoplasma capsulatum, Nocardia asteroides*) and parasites such as roundworm and hookworm survive in the soil and cause human infection.

  Water may act as the source of infection either due to contamination with pathogenic microorganisms (*Vibrio cholerae*, hepatitis A virus) or due to the presence of aquatic vectors (cyclops in guineaworm infection).
- ***Food*** Food may be a source of infection if it contains pathogens, which may result from external contamination (food poisoning by staphylococcus) or pre-existent infection in meat or other animal products (salmonellosis).
- ***Inanimate objects*** This includes penetrating injuries by contaminated objects, accidents, unsterile instruments and equipment used in patient care and fomites.

METHODS OF TRANSMISSION OF INFECTION

1. Contact This may be via:

- **Direct contact** Infections may be acquired by **direct contact**, that is, from person to person, e.g., sexually transmitted diseases such as syphilis and gonorrhea are transmitted by direct contact.
- **Indirect contact** The mode of transmission of some infections may be through the agency of **fomites**, which are inanimate objects such as clothing, bedding, towels, etc.

2. **Inhalation** Respiratory infections such as influenza and tuberculosis are transmitted by inhalation of the pathogen. Such microbes are shed from the patients' nose or throat during sneezing, speaking or coughing.

> Large drops of secretions fall to the ground and dry up. Pathogens resistant to drying may remain viable in the dust and act as sources of infection. Small droplets, under 0.1 mm in diameter, evaporate immediately to become minute particles or **droplet nuclei** (usually 1–10 ∝m in diameter), which remain suspended in the air for long periods and act as sources of infection.

3. **Ingestion** Intestinal infections are generally acquired by the ingestion of food or drink contaminated by pathogens. Such infections may be **waterborne** (cholera), **food-borne** (food poisoning) or borne by unclean hands (dysentery).

4. **Inoculation** Pathogens may be introduced directly into the tissues through a breach in the skin and soft tissue. Some examples are:

- Tetanus spores implanted in deep wounds (following penetrating injury contaminated by soil)
- Rabies virus deposited subcutaneously by a dog bite
- Arboviruses injected by insect vectors

5. **Congenital** Some pathogens can cross the placental barrier and infect the fetus in utero. This is known as **vertical transmission**, e.g., congenital syphilis and intrauterine infection with the rubella virus. Such infections are known as **teratogenic infections**.

6. **Iatrogenic and laboratory infections** Infection by inoculation may be **iatrogenic** (illness caused by medical intervention). Such infections may be caused by:

- Using unsterile syringes and surgical instruments or equipment.
- Transfusion of infected blood or the use of contaminated syringes and needles, particularly among addicts of injectable drugs (e.g., human immunodeficiency virus, i.e., HIV).
    - Exchange transfusion, dialysis and organ transplant surgery, which have increased the possibilities for iatrogenic infections (e.g., hepatitis B and C).
    - Laboratory personnel handling infectious material are also at risk (e.g., anthrax, plague and tuberculosis).

FACTORS PREDISPOSING MICROBES TO PATHOGENICITY

It is important to be familiar with the common terms used in connection with the microorganism's ability to produce infection. Some important terms are as follows:

Pathogenicity It is the ability of a microorganism to produce disease.

Virulence It is the degree of the disease-producing property of the microorganism and vaccine strains of the microorganism. The range of virulence may vary from highly virulent (e.g., ebola virus) to avirulent (e.g., micrococci). The virulence of a strain is not constant and may undergo spontaneous or induced variation.

Exaltation This refers to the enhancement of virulence, which is done in vitro by serial passage in susceptible hosts.

Attenuation It is the reduction of virulence, which is achieved by passage hosts, repeated cultures in artificial media, growth in high temperature or in the presence of weak antiseptics, desiccation or prolonged storage.

Factors Influencing Virulence

Virulence is determined by one or more of the following properties of microorganisms:

1. Adhesion

Attachment of the bacteria to body surfaces is the first step in pathogenesis. This attachment is a specific reaction between surface receptors on host

cells and adhesive structures (ligands) called **adhesins** on the surface of bacteria.

Adhesins may occur as organised structures, such as fimbriae or pili, or as colonisation factors. This specific adhesin may account for the tissue tropisms and host specificity exhibited by many pathogens. Adhesins serve as virulence factors, the loss of which renders the strain avirulent. Adhesins are usually made of protein and are antigenic in nature. Specific immunisation with adhesins has been attempted as a method of prophylaxis in some infections, as for instance against *E. coli* diarrhea in calves and piglets, and gonorrhea in human beings.

2. Invasiveness

This refers to the ability of a pathogen to spread in the host tissues after establishing infection. Highly invasive pathogens spread from the site of infection to other parts of the body (e.g., streptococcal septicemia following localised wound infection). Less invasive pathogens cause localised lesions (e.g., abscess due to staphylococci). Some pathogens, though capable of causing serious or even fatal diseases, lack invasiveness altogether (e.g., *C. tetani* remains confined to the site of entry and produces the disease by elaborating a potent toxin).

3. Toxigenicity

Bacteria produce two types of toxins—exotoxins and endotoxins.

Exotoxins are **heat-labile proteins** which are secreted by certain species of bacteria and diffuse readily into the surrounding medium. They are highly potent in minute amounts and constitute some of the most poisonous substances known. One mg of tetanus or botulinum toxin is sufficient to kill more than one million guinea pigs; it has been estimated that 3 kg of botulinum toxin can kill all the inhabitants of the world. Exotoxins treated with formaldehyde yield **toxoids** that are non-toxic but retain the ability to induce antibodies **(antitoxins**). They exhibit specific tissue affinities and pharmacological activities, each toxin producing a typical effect which can be made out by characteristic clinical manifestations or autopsy appearances. Exotoxins are generally formed by gram-positive bacteria but may also be produced by some gram-negative organisms such as Shiga's strain of *Shigella dysenteriae*, *Vibrio cholerae* and enterotoxigenic *E. coli.*

Endotoxins are **heat-stable lipopolysaccharides (LPS)** which form an integral part of the cell wall of gram-negative bacteria. Their toxicity depends on the lipid component (**lipid A**). They are not secreted outside the

bacterial cell and are released only by the disintegration of the cell wall. They cannot be toxoided. They are poor antigens and their toxicity is not completely neutralised by the homologous antibodies. They are active only in relatively large doses. They do not exhibit specific pharmacological activities. All endotoxins, whether isolated from pathogenic or non-pathogenic bacteria, produce similar effects.

Administration of small quantities of endotoxin in susceptible animals causes an elevation of body temperature within 15 minutes, which may last for several hours. The pyrogenic effect of fluids used for intravenous administration is usually due to the presence of endotoxins from contaminating bacteria. Gram-negative septicemias or intravenous injections of large doses of endotoxin cause endotoxic shock marked by fever, leucopenia, thrombocytopenia, significant fall in blood pressure, circulatory collapse and bloody diarrhea, leading to death (Table 7.1). Clotting of Limulus lysate (lysate of amebocytes from horseshoe crab, *Limulus polyphemus*) is used as a test to detect endotoxins.

Table 7.1 Biological activities of endotoxins

Pyrogenicity	Lethal action	Depression of blood pressure
Activation of complement	Intravascular coagulation	Leucopenia
Leucocytosis	Inhibition of glucose and glycogen synthesis in the liver	Stimulation of B lymphocytes
Macrophage inhibition	Interferon release	Induction of prostaglandin synthesis

Exotoxins differ in their properties and actions from endotoxins (Table 7.2).

Table 7.2 Distinguishing features of exotoxins and endotoxins

Exotoxins	Endotoxins
Proteins	Lipopolysaccharides
Heat-labile	Heat-stable
Actively secreted by cells; diffuse into surrounding medium	Form part of cell wall; do not diffuse into surrounding medium

Readily separable from cultures by physical means such as filtration	Obtained only by cell lysis
Action often enzymic	No enzymic action
Specific pharmacological effect for each exotoxin	Effect non-specific; action common to all endotoxins
Specific tissue affinities	No specific tissue affinity
Active in very minute doses	Active only in very large doses
Highly antigenic	Weakly antigenic
Action specifically neutralised by antibody	Neutralisation by antibody is ineffective

4. Genetic material

Genes coding for virulence factors in bacteria can be harboured in plasmids, which are extra-chromosomal. These plasmids may be transmitted via bacteriophages, which act as vehicles for the passage of plasmids from one bacterium to another.

Plasmids Plasmid-borne genes code for some virulence characteristics, e.g., surface antigens responsible for the colonisation of intestinal mucosa by *E. coli* and enterotoxin production by *E. coli* and *S. aureus* and multiple drug resistance (R) plasmids, which increase the severity of clinical disease by producing resistance to antibiotic therapy.

Bacteriophages The classical example of phage-directed virulence is seen in diphtheria. In the diphtheria bacilli, the gene for toxin production is present in beta or other tox^+ corynephages.

5. Communicability

The ability of a parasite to spread from one host to another is known as communicability. **This property does not influence the production of disease in an individual host but determines the survival and distribution of a parasite in a community**. A correlation need not exist between virulence and communicability.

A highly virulent parasite may not exhibit a high degree of communicability. In general, infections in which the pathogen is shed in secretions, as in respiratory or intestinal diseases, are highly communicable. In some instances, as in rabies, human infection represents a dead end, as the infection does not spread from one human to another (although in rare

cases, it may be transmitted by organ transplant or human bite).

Development of epidemic and pandemic diseases requires the pathogenic strain to possess a high degree of virulence and communicability.

6. Miscellaneous

There are several other factors that influence and favour the establishment of infection by a microorganism.

i. Bacterial products

Some bacterial products other than toxins, though devoid of intrinsic toxicity, may contribute to virulence by inhibiting the mechanisms of host resistance. They are as follows:

Coagulase—a thrombin-like enzyme which prevents phagocytosis by forming a fibrin barrier around the bacteria and walling off the lesion (e.g., *Staphylococcus aureus*).

Fibrinolysins—promote the spread of infections by breaking down the fibrin barrier in tissues.

Hyaluronidases—split hyaluronic acid, which is a component of intercellular connective tissue, thus facilitating the spread of infection along tissue spaces.

Leucocidins—damage polymorphonuclear leucocytes.

Hemolysins—capable of destroying erythrocytes.

ii. Bacterial appendages

Capsulated bacteria such as pneumococci, *K. pneumoniae* and *H. influenzae* are not readily phagocytosed. Some bacterial surface antigens such as the Vi antigen of *S.* Typhi and K antigens of *E. coli* also help the bacteria to withstand phagocytosis and the lytic activity of complements.

iii. Biofilms

These are well-organised microcolonies of bacteria enclosed in self-produced extracellular polymer matrices known as glycocalyx. They are separated by water channels that remove water and deliver nutrients. They may be classified as adherent, clinging and submerged biofilms.

Biofilms are of two types: **monomicrobial biofilms** and **polymicrobial biofilms**.

Free-floating bacteria come in contact with medical devices and attach to them with pili. They then aggregate, multiply and secrete extracellular polymers and, in turn, get encased under the polymers. These polymers act as a barrier to the action of antibiotics. Prevention of biofilm formation in medical devices is done by using sonication, antibiotics, catheter lock solutions and catheter flushing.

iv. Infecting dose

Successful infections require that an adequate number of bacteria gain entry into the host. This dosage may be estimated as the **minimum infecting dose** (MID) (i.e., the minimum number of bacteria required to produce clinical evidence of infection) or **minimum lethal dose** (MLD) (i.e., the minimum number of bacteria required to cause death of a susceptible animal under standard conditions). As animals exhibit considerable individual variation in susceptibility, these doses are more correctly estimated in statistical expressions as ID50 (infectious dose 50) and LD50 (lethal dose 50)—the dose required to infect or kill 50 per cent of the animals tested under standard conditions.

v. Route of infection

Some bacteria, such as streptococci, can initiate infection whatever be the mode of entry. Others can survive and multiply only when introduced by the optimal routes.

V. cholerae is infective orally but is unable to cause infection when introduced subcutaneously. Bacteria also differ in their sites of selection in the host body after introduction into tissues (organ- or tissue-specific).

Bacteremia refers to the circulation of bacteria in the blood. Transient bacteremia is a frequent event even in healthy individuals and may occur during chewing, brushing of teeth or straining at stools. These bacteria are immediately mopped up by phagocytic cells and are unable to initiate infection. Bacteremia of greater severity and longer duration is seen during generalised infections as in typhoid fever.

Septicemia is the condition where bacteria circulate and multiply in the blood, form toxic products and cause fever.

Pyemia is a bloodstream infection caused by a pyogenic organism (e.g., *Staphylococcus*) following an abscess or focal, pyogenic infection.

CLASSIFICATION OF INFECTIOUS DISEASES

Depending on their spread in the community, infectious diseases may be classified into different types:

- An **endemic infection** is one that is constantly present in a particular area. Typhoid and malaria are endemic in most or some parts of India.
- An **epidemic** infection is one that spreads rapidly, involving many persons in an area at the same time. Influenza and dengue cause annual epidemics.
- A **pandemic** is an epidemic that spreads across many countries of the world, involving very large numbers of people within a short period. Influenza is a pandemic infection.
- A **prosodemic** infection is a slow-spreading infection transmitted from person to person.

The outcome of an infection depends on the host–pathogen interaction (Fig. 7.2).

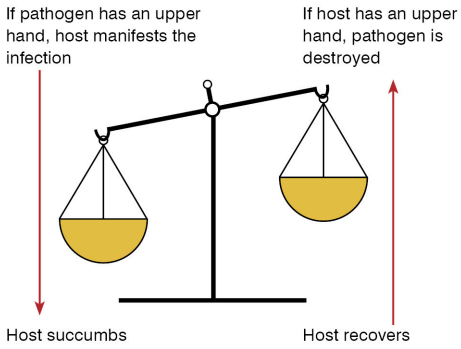

If pathogen has an upper hand, host manifests the infection
If host has an upper hand, pathogen is destroyed
Host succumbs
Host recovers

Fig. 7.2 Outcome of host–pathogen interaction

IMMUNITY

The term ‘immunity’ refers to the response of the host towards injury caused by microorganisms or any foreign substance. Protection against infectious diseases is only one of the many consequences of an immune response.

The types of immunity are laid out in the form of a flowchart in Fig. 7.3 and elaborated in the following sections.

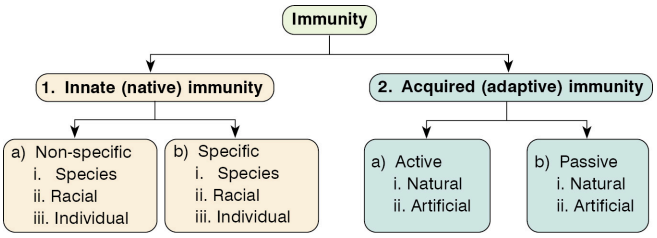


Fig. 7.3 Types of immunity

INNATE OR NATIVE IMMUNITY

The resistance to infections that an individual possesses by virtue of his genetic and constitutional make-up is referred to as innate immunity. It does not require prior contact with microorganisms or immunisation. It may be non-specific (when it indicates a degree of resistance to infections in general) or specific (where resistance to a particular pathogen is concerned).

Innate immunity may be considered at the level of the species, race or individual.

Species immunity This refers to the total or relative refractoriness to a pathogen, shown by all members of a species. Humans are non-susceptible to plant pathogens and to many animal pathogens such as rinderpest and distemper. The mechanisms of species-specific immunity are not clearly understood but may be due to physiological and biochemical differences between the tissues of the different host species.

Racial immunity Within a species, different races may show differences in susceptibility to infection. The high resistance of Algerian sheep to anthrax is an example. Such racial differences are known to be genetic in origin, and are shaped by selection and inbreeding. An interesting instance of genetic resistance to *Plasmodium falciparum* malaria is seen in some parts of Africa and the Mediterranean coast. A hereditary abnormality of red cells (sickling) prevalent in the area confers immunity to infection by the malarial parasite.

Individual immunity This is the difference in innate immunity exhibited by different individuals in a race. It is documented that homozygous twins exhibit similar degrees of resistance or susceptibility to lepromatous leprosy and tuberculosis. Such correlation is not seen in heterozygous twins.

Factors Affecting Innate Immunity

- ***Age*** The very young and the very old are more susceptible to infectious diseases than the rest.

> The fetus in utero is normally protected from maternal infection by the placental barrier (except from infections like rubella, herpes, cytomegaloviruses and *Toxoplasma gondii*, which lead to congenital malformations; these pathogens can cross the placental barrier).

- ***Hormonal influences*** Endocrine disorders such as diabetes mellitus, hypothyroidism and adrenal dysfunction are associated with enhanced susceptibility to infection. The high incidence of staphylococcal sepsis in diabetes may be related to the increased level of carbohydrates in the tissues. Corticosteroids exert an important influence on the host's response to infection. They depress the host's resistance by their anti-inflammatory and anti-phagocytic effects and by the suppression of antibody formation and hypersensitivity. Elevated steroid levels during pregnancy may be related to the heightened susceptibility of pregnant women to many infections.
- ***Nutrition*** The relationship between malnutrition and immunity is complex. In general, both humoral and cell-mediated immune processes are reduced when there is malnutrition. **The Mantoux test is negative for cell-mediated immune response in severe protein deficiency, as in kwashiorkor.**

Mechanisms of Innate Immunity

Conserved microbe-specific molecules, referred to as microbe-associated or pathogen-associated molecular patterns (MAMPs or PAMPs), are recognised by the plant's innate immune system's pattern recognition receptors (PRRs). These are plant-specific.

Intact physical barriers constitute an important component of non-specific innate immunity.

Epithelial surfaces

- The intact **skin** and **mucous membrane** covering the body protect it considerably against the invasion of microorganisms. Healthy skin possesses bactericidal activity due to the presence of a high **concentration of salt in sweat and sebaceous secretions**. Persons who immerse their hands in soapy water for long periods have frequent mycotic and pyogenic infections due to the decrease in bactericidal activity of skin secretions caused by soap. However, resident flora are not easily removed even by washing and application of disinfectants.
- **Mucosa** of the respiratory tract has several innate mechanisms of defence. Inhaled particles are arrested at or near the nasal orifices. Those that pass beyond are held by the **mucus lining of the epithelium** and are swept back to the pharynx, where they tend to be swallowed or coughed out. The **cough reflex** is an important defence mechanism of the respiratory tract. The **cilia** on the respiratory epithelial cells propel particles upwards. Nasal and **respiratory secretions contain mucopolysaccharides** capable of combining with influenza and certain other viruses. Particles that manage to reach the pulmonary alveoli are ingested by the **phagocytic cells** present there.
- **Saliva,** which bathes the mouth**,** has an inhibitory effect on many microorganisms. Particles deposited in the mouth are swallowed and subjected to the action of the **digestive juices**. The **highly acidic gastric juice** of the stomach destroys most microorganisms. The **conjunctiva** is freed of foreign particles by the flushing action of **lachrymal secretions**. The eyes become susceptible to infection when lachrymal secretions are absent. **Tears** contain the antibacterial substance **lysozyme,** first described by Fleming (1922). This is a thermolabile, low-molecular-weight, basic protein which acts as a muraminidase.

> **Lysozyme** is present in tissue fluids and in nearly all secretions except cerebrospinal fluid, sweat and urine. It acts by splitting certain polysaccharide components of the cell walls of susceptible bacteria. In the concentrations seen in tears and other secretions, lysozyme is active only against some non-pathogenic gram-positive bacteria. However, it occurs in phagocytic cells in concentrations high enough to be lethal to many pathogens.

- The flushing action of urine removes bacteria.
- **Spermine and zinc** present in semen have antibacterial activity.
- The **acidic pH of the adult vagina,** due to the fermentation of glycogen in the epithelial cells by the resident aciduric bacilli, makes it inhospitable to many pathogens.

Antibacterial substances in blood and tissues

The complement system possesses bactericidal activity and plays an important role in the destruction of pathogenic bacteria that invade the blood and tissues. Several substances possessing antibacterial properties have been described in blood and tissues. Some of these substances are as follows:

- **Beta-lysin**, a relatively thermostable substance active against anthrax and related bacilli
- **Basic polypeptides**, such as leukins extracted from leucocytes and plakins from platelets
- **Acidic substances**, such as lactic acid found in muscle tissue and in the inflammatory zones
- **Lactoperoxidase** in milk possesses antibacterial properties demonstrable experimentally; however, their relevance in the natural context is not clearly understood
- **Interferon**, which has been shown to be more important than specific antibodies in the protection against and recovery from certain acute viral infections

Tissues and body secretions contain other antiviral substances.

Microbial antagonisms

The skin and mucous surfaces have resident bacterial flora which prevent colonisation by pathogens. Alteration of the normal resident flora may lead to invasion by extraneous microbes, causing serious diseases such as staphylococcal or clostridial enterocolitis following oral antibiotics. **Germ-free animals are extremely susceptible to all types of infections due to the absence of normal bacterial flora.**

Cellular factors

Phagocytic cells which ingest and destroy microorganisms and other foreign particles were discovered by Metchnikoff in 1883.

- **Microphages,** which are polymorphonuclear leucocytes, get rid of any possibly harmful microorganisms and other unwanted debris in the cells.
- **Macrophages,** which include monocytes, histiocytes and fixed reticuloendothelial cells, detect, engulf and destroy pathogens and apoptotic cells. Macrophages are produced through the differentiation of monocytes.
- **Mast cells** release granules and powerful chemical mediators such as histamine, cytokines, granulocyte macrophage colony-stimulating factor (GM-CSF), leukotrienes, heparin and many proteases into the environment.
- **Dendritic cells** are antigen-presenting cells which are found in tissues and skin. They are primary to the innate immune system.
- **Mucosa-associated lymphoid cells** are abundant in the gut, including the oral and rectal mucosa. They have specialised pathways for antigen uptake.

Natural defence against the invasion of blood and tissues is mediated by phagocytic cells. A major function of the reticuloendothelial system is the removal of foreign particles that enter the body. Phagocytic cells reach the sites of inflammation in large numbers, attracted by **chemotactic substances**, and ingest particulate material.

> Capsulated bacteria, such as *S. pneumoniae*, are not readily phagocytosed except in the presence of **opsonins**. They are more readily phagocytosed when trapped against a firm surface such as the alveolar wall than when they are free in tissue fluids.

Natural killer cells (NK cells) are a class of lymphocytes important in non-specific defence. They selectively kill virus-infected cells and tumour cells. NK cells are activated by interferons.

Phagocytosis

Bacteria are phagocytosed into a vacuole **(phagosome**), which fuses with

the **lysosomes** found in the cell to form the **phagolysosome** (Fig. 7.4a and b). The bacteria are subjected to the action of the lytic enzymes in the phagolysosome and are destroyed.

Some bacteria, such as brucella and lepra bacilli, resist intracellular digestion and may actively multiply inside the phagocytic cells. Phagocytosis in such instances may actually help to disseminate infection to different parts of the body. The importance of phagocytosis in the protection against infection is evidenced by the enhanced susceptibility to infection seen either when the phagocytic cells are depleted, as in agranulocytosis, or when they are functionally deficient, as in chronic granulomatous disease.

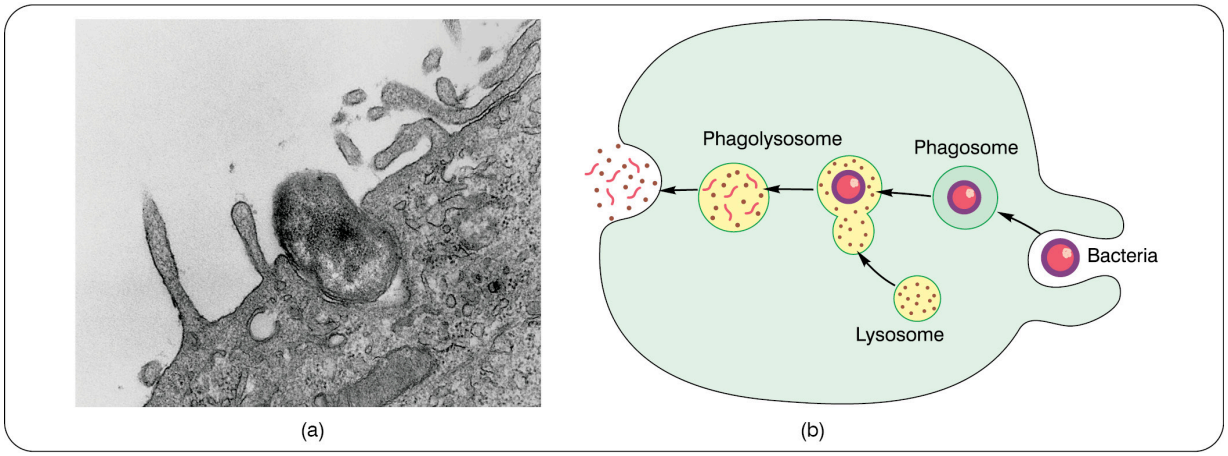


Fig. 7.4 (a) Transmission electron microscopic (TEM) picture of *Rickettsia tsutsugamushi* getting phagocytosed by mouse mesothelial cell and (b) diagrammatic representation of phagocytosis (*Source:* (a) Public Health Image Library, ID 923 / Dr. Edwin P. Ewing, Jr / CDC)

Inflammation

This is a non-specific defence mechanism. The arterioles at the site of injury constrict initially and then dilate, leading to increased blood flow. This is followed by the slowing of blood flow and margination of the leucocytes, which escape into the tissues by diapedesis and accumulate in large numbers, attracted by the chemotactic substances released at the site of injury. As a result, microorganisms around the site are phagocytosed and destroyed. There is an outpouring of plasma, which helps to dilute the toxic products present. A fibrin barrier is laid, serving to wall off the site of infection.

Fever

A rise in temperature following infection is a natural defence mechanism. It not only helps to accelerate the physiological processes but may also, in some cases, actually destroy the infecting pathogens. Fever stimulates the production of interferons and aids in the recovery from viral infections.

> Historically, therapeutic induction of fever was used for the destruction of *Treponema pallidum* in the tissues of syphilitic patients before penicillin became available.

Acute-phase reactant proteins

Infection or injury leads to a sudden increase in the plasma concentration of certain proteins, collectively called acute-phase proteins. These include:

- C-reactive protein (CRP), an acute-phase reactant (APR) that accompanies inflammation and tissue injury. It was first observed in the serum of patients during the acute phase of pneumococcal pneumonia; hence the name CRP. The level of this protein in the serum is maintained by homeostatic mechanism. It can change substantially during an acute phase of infection. This is commonly used in clinical practice as an adjunct diagnostic test in bloodstream infections, especially in the pediatric population.
- Mannose-binding protein
- Alpha-1-acid glycoprotein
- Serum amyloid
- P component

CRP and some other acute-phase proteins activate the alternative pathway of complements. They are believed to enhance host resistance, prevent tissue injury and promote the repair of inflammatory lesions.

Toll-like receptors

Many of the molecules involved in innate immunity have the property of pattern recognition, i.e., the ability to recognise a given class of molecules. Certain molecules are unique in microbes and are not found in multicellular organisms. To recognise these patterns and to destroy the invaders displaying such molecules is a strong feature of innate immunity. Such molecules are a class of cell-associated receptors known as toll-like receptors (TLRs). There are 13 different TLRs that recognise pathogens,

enhance phagocytosis and lead to inflammation at the site.

The factors contributing to innate immunity are listed in Table 7.3.

Table 7.3 Non-specific host defence barriers contributing to innate immunity

Body systems and factors involved	Active component	Effector mechanism
Skin	Squamous cells, salt of sweat glands, sebaceous glands (long-chain fatty acids and lactic acid)	Desquamation, flushing, organic acids, bactericidal pH (3–5) retards bacterial growth
Mouth	Saliva	Bathing, flushing
GI tract	Wall, gastric secretions	Peristalsis, mucus, low pH, flushing
Respiratory tract	Tracheal cilia, cough reflex	Mucociliary elevator, surfactant, forcible removal
Nasopharynx	Nasal hair, mucus	Mechanical barrier, entrapment and swallowing
Eye	Tears	Flushing, lysozyme
Circulation and lymphoid organs	Phagocytic cells, NK cells	Phagocytosis, intracellular killing, direct and antibody-dependent cytolysis
Serum	Lactoferrin and transferrin, interferons, TNFα, lysozyme, fibronectin, complement proteins	Iron binding, antiviral proteins, phagocyte activation, peptidoglycan hydrolysis, opsonisation and phagocytosis, enhanced intracellular killing
Cell-associated receptors	Toll-like receptors	Pattern recognition of pathogen and elimination

Acute-phase proteins	CRP, mannose-binding protein, alpha-1-acid glycoprotein	Inflammation
Adult vagina	Acidic pH	Retard bacterial growth
Semen	Spermine and zinc	Antibacterial activity
Urinary system	Acidic pH and flow	Retard bacterial growth, flushing
Breast milk	Antibodies, fibronectin, interferon, lactoferrin, lysozyme, mucin, oligosaccharides	Protection of newborn
Fever	High temperature	Destroys the pathogen

Acquired or Adaptive Immunity

The resistance that an individual acquires during life by recognising and selectively eliminating specific foreign molecules is known as **acquired immunity**. This form of immunity displays four characteristic features:

1. ***Antigenic specificity*** The immune system or antibodies can distinguish among antigens, even between two proteins that differ in only one amino acid.
2. ***Diversity*** The immune system is capable of generating enormous antibody diversity in its recognition molecules, allowing it to reorganise billions of unique structures/patterns on foreign antigens. Genes form the basis of such diversity.
3. ***Immunologic memory*** The immune system exhibits memory on the second encounter of the same antigen by generating a secondary response which is more specific, heightened and quick.
4. ***Self/non-self recognition*** Self-tolerance is one of the unique characteristics of the immune system, which prevents it from reacting to the body's own molecules while still effectively eliminating foreign antigens. Multiple mechanisms ensure self-tolerance. Failure of these mechanisms may lead to autoimmunity.

Types of Acquired Immunity

Acquired immunity may be broadly of two types: active and passive.

Active immunity

Active immunity is the resistance developed by an individual in response to an antigenic stimulus. It is also known as **adaptive immunity** as it represents the adaptive response of the host to a specific pathogen or other antigen. The important features of active immunity are as follows:

- It involves the active functioning of the host's immune apparatus, leading to:
  a) synthesis of **antibodies (humoral immunity)**
  b) production of **immunologically active cells** (**cellular immunity**)
- It sets in only after a latent period which is required for the immunological machinery to be set in motion.
- During the development of active immunity, there is often a negative phase during which the level of measurable immunity may actually be lower than it was before the antigenic stimulus. This is because the antigen combines with any pre-existing antibody and lowers its level in circulation.
- Once developed, active immunity is long-lasting. If an individual who has been actively immunised against an antigen is exposed to the same antigen subsequently, the immune response is faster and more abundant than it was during the first encounter. This is known as **secondary response**.
- Besides the development of humoral and cellular immunity, active immunity is associated with **immunological memory**.
- Active immunisation is more effective and confers better protection than passive immunisation.

There are two **types of active immunity—natural active immunity and artificial active immunity.**

Natural active immunity

Natural active immunity results from either a clinical or an inapparent infection by a microbe.

A person who has recovered from a bout of measles develops natural active immunity. The large majority of adults in the developing countries possess natural active immunity to poliomyelitis due to repeated inapparent

infections in childhood. Such immunity is usually long-lasting but the duration varies with the type of pathogen. The acquired immunity to viral diseases such as chickenpox and measles is usually lifelong. In some diseases, such as influenza or the common cold, the immunity appears to be short-lived.

> Influenza can recur in an individual after a few months or a year but this is not so much due to the lack of the immunising capacity of the virus but to its ability to undergo antigenic variation. Immunity following the first infection is not effective against the second infection, which is caused by an antigenically variant virus.
> On the other hand, in the common cold, the apparent lack of immunity is because the same clinical picture can be caused by infection with a large number of different viruses.

The immunity following bacterial infection is generally less permanent than that following viral infection. In syphilis, a special type of immunity known as '**premunition**' is seen. Here, immunity to re-infection lasts only as long as the original infection remains active. Once the disease is cured, the patient becomes susceptible to the spirochete once again. In chancroid, another venereal disease caused by *Haemophilus ducreyi*, there does not appear to be any effective immunity as the patient may develop lesions following re-infection even while the original infection is active.

Artificial active immunity

Artificial active immunity is the resistance induced by vaccines. **Vaccines are preparations of live or killed microorganisms or their products used for immunisation**. Examples of vaccines are as follows:

Bacterial vaccines

- Live (BCG vaccine for tuberculosis)
- Killed (Cholera vaccine)
- Subunit (Typhoid Vi antigen)
- Bacterial products (Tetanus toxoid)

Viral vaccines

- Live (Oral polio vaccine—Sabin)
- Killed (Injectable polio vaccine—Salk)
- Subunit (Hepatitis B vaccine)

Live (attenuated) vaccines The immunity following live vaccine administration parallels that following natural infection though the former initiates a lower order infection without causing any injury or disease. The immunity lasts for several years but booster doses may be necessary. Live vaccines may be administered orally (as with the Sabin vaccine for poliomyelitis) or parenterally (as with the measles vaccine).

Killed vaccines These are generally less immunogenic than live vaccines, and the protection conferred by them lasts only for a short period. They have, therefore, to be administered repeatedly, generally at least two doses being required for the production of immunity. The first dose is known as the **primary dose** and the subsequent doses as **booster doses**. Killed vaccines may be given orally but this route is generally not effective. Parenteral administration elicits a humoral antibody response, which may be improved by the addition of an '**adjuvant**' (e.g., aluminium phosphate).

Passive immunity

This is the resistance that is transmitted passively to a recipient in a 'readymade' form. Here, the recipient's immune system plays no active role. The features of passive immunity are as follows:

- There is no antigenic stimulus; instead, pre-formed antibodies are administered.
- There is no latent period, protection being effective immediately after passive immunisation.
- There is no negative phase. The immunity is transient, usually lasting for days or weeks, only till the passively transmitted antibodies are metabolised and eliminated.
- No secondary type response occurs in passive immunity.

In fact, passive immunity diminishes in effect with repetition. When a foreign antibody is administered a second time, it is eliminated more rapidly than it was initially.

Following the first injection of an antibody such as immune horse serum, its elimination is only by metabolic breakdown but during subsequent injections of horse serum, elimination is much quicker as it combines with antibodies to horse serum that would have been produced following its initial injection. This factor of **immune elimination** limits the usefulness of repeated passive immunisation.

There are two **types of passive immunity—natural passive immunity and artificial passive immunity.**

Natural passive immunity

Natural passive immunity is the immunity that is passively transferred from a mother to her baby. In human infants, maternal antibodies are transmitted through the placenta. The human colostrum, which is rich in IgA antibodies resistant to intestinal digestion, gives protection to the neonate. By active immunisation of mothers during pregnancy, it is possible to improve the quality of passive immunity in their infants. Immunisation of pregnant women with tetanus toxoid is recommended for this purpose in communities in which neonatal tetanus is common.

The human fetus acquires some ability to synthesise antibodies (IgM) from about the twentieth week of life but its immunological capacity is still inadequate at birth. It is only by the age of about three months that the infant acquires some measure of immunological independence. Until then, maternal antibodies give passive protection against infectious diseases to the infant. It is for this reason that most pediatric infections are more common after the age of three months than in younger infants.

Artificial passive immunity

Artificial passive immunity is the resistance passively transferred to a recipient by the administration of antibodies. Substances that provide a host with such immunity are as follows:

- **Hyperimmune sera of human or animal origin** such as human hyperimmune globulin [e.g., tetanus immune globulin (TIG) and antitetanus serum (ATS) obtained from hyperimmunised horses]
- **Convalescent sera**—sera of patients recovering from infectious diseases contain high levels of specific antibodies (e.g., viral hepatitis A)
- **Pooled human gamma globulin** (gamma globulin from pooled sera of healthy adults) contains antibodies against all common pathogens prevalent in the region; human gamma globulin is also used in the treatment of patients with some immunodeficiencies

Antisera of animal origin are now recommended only where human preparations are not available (anti-gas gangrene and anti-botulinum sera; antivenoms).

Indications Passive immunisation is indicated for immediate and temporary protection in a non-immune host faced with the threat of infection. Passive immunisation may also be used for the suppression of active immunity, when the latter may be injurious. An example is the use of Rh immune globulin during delivery to prevent immune response to the Rhesus factor in Rh-negative women pregnant with Rh-positive babies.

Passive immunisation is less effective than active immunisation. The main advantage of passive immunisation is that it acts immediately and, therefore, can be employed when **'instant' immunity** is desired. Table 7.4 presents the differences between active and passive immunity.

Table 7.4 Comparison of active and passive immunity

Active immunity	Passive immunity
Produced actively by host's immune system	Received passively; no active host participation
Induced by infection or by immunogens	Readymade antibody transferred
Durable, effective protection	Transient, less effective
Immunity effective only after lag period	Immediate immunity
Immunological memory present	No memory
Booster effect on subsequent dose	Subsequent dose less effective
Negative phase may occur	No negative phase
Not applicable in the immunodeficient	Applicable in the immunodeficient

Combined immunisation

Sometimes, a combination of the active and passive methods of immunisation is used to protect an individual against a certain disease. Ideally, whenever passive immunisation is employed for immediate protection, combined immunisation is preferred, as in the protection of a non-immune individual with a tetanus-prone wound. The method involves the injecting of TIG in one arm and the first dose of tetanus toxoid in the other. This is followed by the full course of phased tetanus toxoid injections. TIG provides the protection necessary till active immunity is

able to take effect.

Adoptive immunity

A special type of immunisation is the injection of immunologically competent lymphocytes. This is known as adoptive immunity and does not have general application. Instead of whole lymphocytes, an extract of immunologically competent lymphocytes, known as the '**transfer factor**', can be used. This has been attempted in the treatment of certain types of diseases (e.g., lepromatous leprosy).

Local immunity

In poliomyelitis, systemic immunity provided by active immunisation with the killed vaccine neutralises the virus when it enters the bloodstream but it does not prevent multiplication of the virus at the site of entry (the gut mucosa). This is achieved by the local intestinal immunity acquired either as a result of natural infection or immunisation with the live oral vaccine. In influenza, immunisation with the killed vaccine elicits a humoral antibody response. But the antibody titre in respiratory secretions is often not high enough to prevent infection. Natural infection or the live virus vaccine administered intranasally provides local immunity. A special class of **immunoglobulins (IgA)** forms the main component of local immunity. **Secretory IgA** is produced locally by plasma cells present on mucosal surfaces or in secretory glands. This indicates the existence of a common **mucosal** or **secretory immune system**.

Herd immunity

This refers to the overall level of immunity in a community and is relevant in the control of epidemic diseases. When a large proportion of individuals in a community (herd) are immune to a pathogen, the herd immunity is satisfactory. When the herd immunity is low, epidemics are likely due to the presence of large numbers of susceptible individuals in the community. Eradication of communicable diseases depends on the development of a high level of herd immunity rather than on the development of a high level of immunity in individuals.

MEASUREMENT OF IMMUNE RESPONSE

The truly valid measurement of immunity is the resistance of an individual to a challenge by the pathogen. This is, however, not valid as the challenge itself alters the state of immunity. It is, therefore, not possible to measure accurately the level of immunity in an individual. Estimates of immunity are generally made by statistical methods using large numbers of individuals.

A simple method of testing immunity is to relate its level to some convenient indicator, such as **demonstration of the specific antibody**. This is not always reliable as the immune response to a pathogen consists of the presence of antibodies to several antigens present in it as well as the production of cellular immunity. Antibodies may be demonstrated by a variety of techniques such as:

- Agglutination
- Precipitation
- Complement fixation
- Hemagglutination inhibition
- Neutralisation

Immunoassays including enzyme-linked immunosorbent assay (ELISA) and its modifications are discussed in detail in later chapters.

In the absence of exact information as to which antigen of the pathogen constitutes the **protective antigen**, serological attempts to measure immunity are only an approximation. Where protection is associated with cell-mediated immunity, skin tests for delayed hypersensitivity and in vitro tests for CMI provide an indication of immunity. In some instances, as in diphtheria, where pathogenesis is due to a well-defined antigen (the toxin), the level of immunity can be assayed by in vitro or in vivo (Schick test) methods. However, this is no longer practised and has become obsolete.

Key points

- Infection and immunity involve the interaction between a host and the infecting microorganism.
- Parasites are microbes that can establish themselves and multiply in hosts.
- Infections may be classified as primary infection, re-infection, secondary infection, focal infection, cross-infection, endogenous infection and inapparent infection.

• Sources of infection:

- Humans constitute the commonest source of infection. The parasite may originate from a patient or a carrier. A carrier may be a healthy carrier, convalescent carrier, a contact carrier or a paradoxical carrier. The carrier state may be temporary or chronic.
- Animals may serve as sources of human infection since the pathogen may be capable of infecting both humans and animals. Infectious diseases transmitted from animals to humans are called zoonoses.
- Bloodsucking insects (mosquitoes, ticks, mites, flies, fleas, lice) that transmit pathogens to humans (arthropod-borne diseases) are called vectors.
- Some pathogens can survive in the soil for very long periods.
- The presence of pathogens in food may be due to external contamination or due to pre-existing infection in meat and other animal products (salmonellosis).

• Infection may be transmitted by direct contact or through fomites, inhalation of droplets, ingestion of food or drink contaminated by pathogens, or directly inoculated into the tissues of the host.

• Pathogenicity is the ability of a microbial species to cause disease, while virulence is the same property in a microbial strain.

• Virulence refers to the degree of the disease-producing property of the microorganism which is determined by:

- The ability of the organism to adhere to surface receptors, the ability to spread in the host tissues and produce toxins, the presence of plasmid-borne or bacteriophage-borne genes, and the ability of the organism to spread from one host to another.
- Microbial products other than toxins that contribute to virulence include coagulase, fibrinolysins, hyaluronidase and leucocidins.
- Microbial appendages such as capsules and certain surface antigens help the bacteria resist phagocytosis and the lytic activity of complements.
- Biofilms are well-organised microcolonies of bacteria enclosed in self-produced extracellular polymer matrices known as glycocalyx.

• The term immunity refers to the resistance exhibited by the host towards injury caused by foreign antigens/microorganisms and their products.

• The two fundamental types of immunity are innate and acquired. Each type is, in turn, composed of different subtypes.

• Innate (native) immunity is the resistance to infection that an individual possesses due to his genetic and constitutional make-up.

• Important non-specific antimicrobial factors found in blood, secretions and tissues include lysozymes, beta-lysin, peroxidase enzymes, interferons and lactoferrin.

• The resistance acquired by an individual during life is known as acquired immunity. It displays four characteristic features: antigenic specificity, diversity, immunologic memory and self/non-self recognition.

• Acquired immunity is of two types: active immunity, where the host produces its own response to foreign antigens to confer protection, and passive immunity, where pre-formed antibodies are

introduced parenterally (not by the oral route but by the intramuscular or intravenous route) into the host to confer protection.

• Herd immunity is the immunity developed in a large proportion (80 per cent) of individuals in a population. This reduces the likelihood of epidemics arising in that community by the pathogen.

Questions

Essays

1. Describe various microbial factors determining the establishment of an infection in a host.
2. Give an account of the types of innate immunity and the factors that affect them.

Short Answers

1. Methods of transmission of infection
2. Factors contributing to microbial pathogenicity
3. Differences between exotoxins and endotoxins
4. Bacterial virulence
5. Various types of carriers
6. Differences between active and passive adaptive immunity
7. Natural and artificial active immunity

Part II: Immunology

Chapter 8

Structure and Functions of the Immune System

Chapter navigation

- Anatomy of immune apparatus
- Cells of immune response
- Major histocompatibility complex

INTRODUCTION

Specific organs are responsible for the development and maturation of cells of the immune system, which are found throughout the body. There are two major types of organs involved with these functions:

- **Primary lymphoid organs,** where the lymphoreticular cells develop and mature
- **Secondary lymphoid organs,** where lymphoid cells and antigens react

The **lymphoreticular system** (or mononuclear phagocyte system) is a complex organisation of cells of diverse morphology distributed widely in different organs and tissues of the body namely blood, connective tissue, spleen, liver, lungs, bone marrow and lymph nodes. They are responsible for immunity. These organs are made up of lymphoreticular cells which consist of lymphoid and reticular components. The mononuclear phagocyte system consists of cells with clearly demarcated functions:

- **Lymphoid cells**—lymphocytes and plasma cells—are primarily concerned with specific immune response.

- **Phagocytic cells**—monocytes and macrophages (otherwise known as scavenger cells)—are primarily concerned with ingesting and digesting exogenous antigens and foreign particles. They contribute to non-specific immunity by removing microorganisms from blood and tissues. They also play a role in antigen processing and specific immunity in the afferent and efferent limbs of the immune response.

Types of Immune Response

The functional anatomy of the lymphoid system enables it to be involved in two types of immune response to an antigen:

- ***Humoral immunity (antibody-mediated)*** Humoral immunity is mediated by antibodies produced by plasma cells present in blood and other body fluids ('humoral' is derived from *humors* meaning 'body fluids').
- ***Cellular immunity*** This form of immunity is mediated by sensitised (activated) lymphocytes.

Cells for each of these components (T and B cells) develop through separate channels and remain independent, though they also interact in some instances (Fig. 8.1).

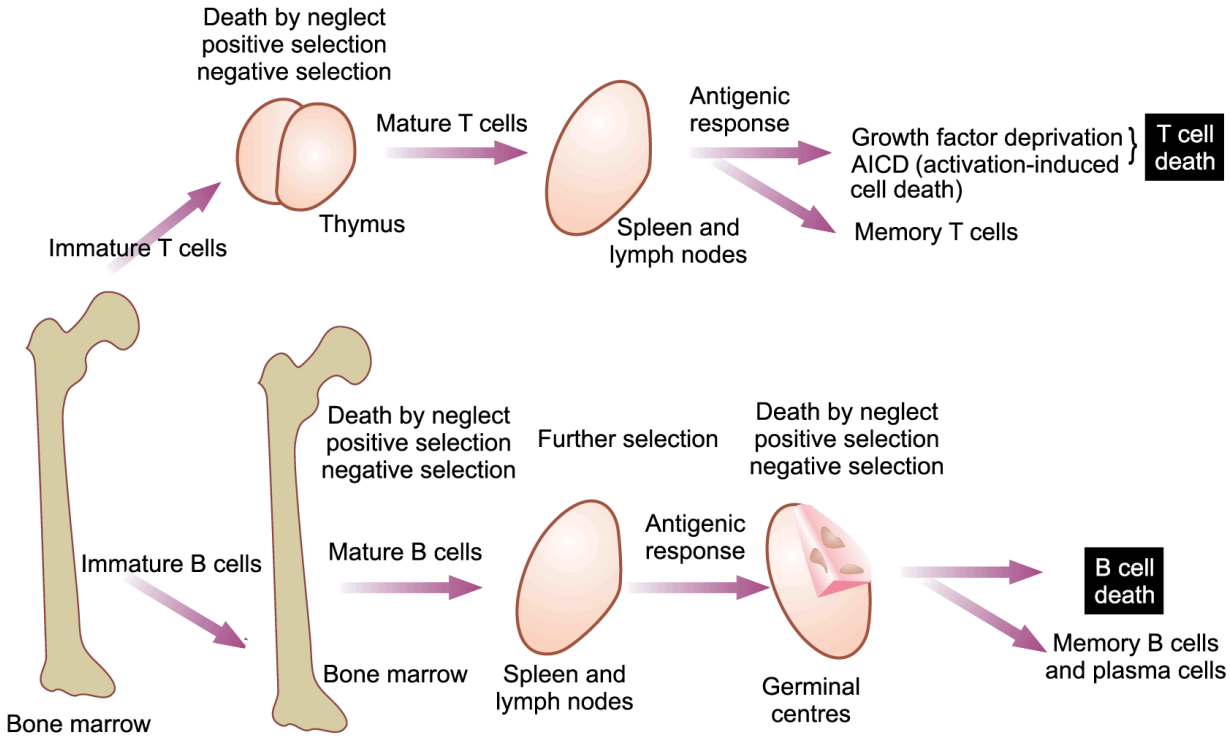


Fig. 8.1 Development and demise of T and B cell systems

- ***Hematopoiesis*** All cells of immunological importance originate from a hematopoietic stem cell (HSC). The details of hematopoiesis are shown in Fig. 8.2.

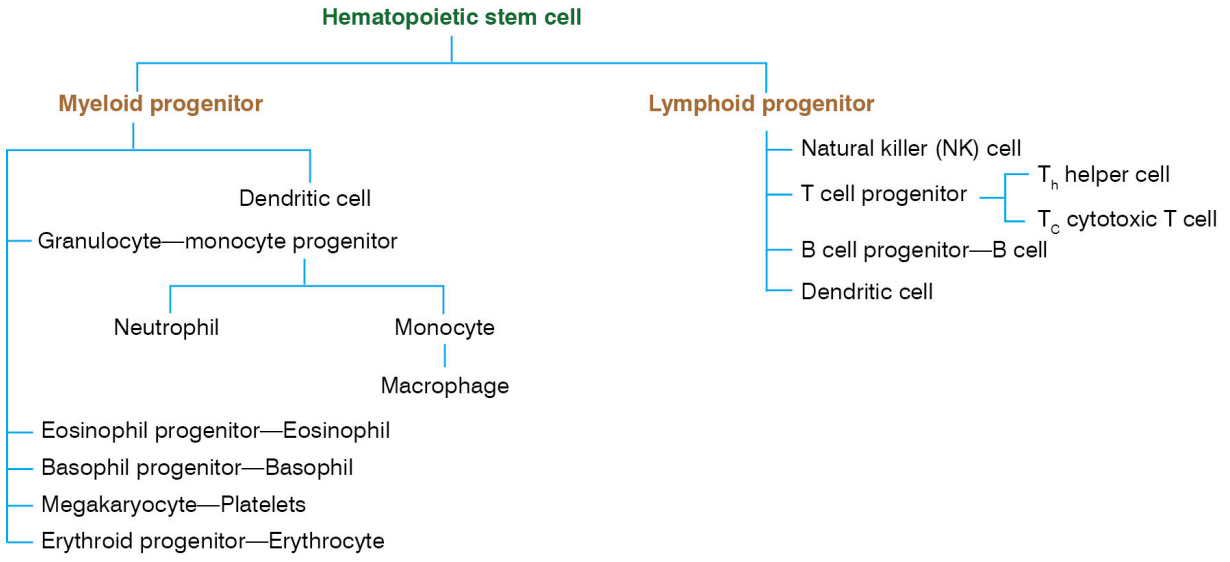


Fig. 8.2 Hematopoiesis

LYMPHOID SYSTEM

The lymphoid system consists of lymphoid organs and cells. The thymus and bone marrow are the **primary lymphoid organs**; the spleen and lymph nodes are the **secondary lymphoid organs**. **Lymphoid cells** consist of lymphocytes and plasma cells (Fig. 8.3).

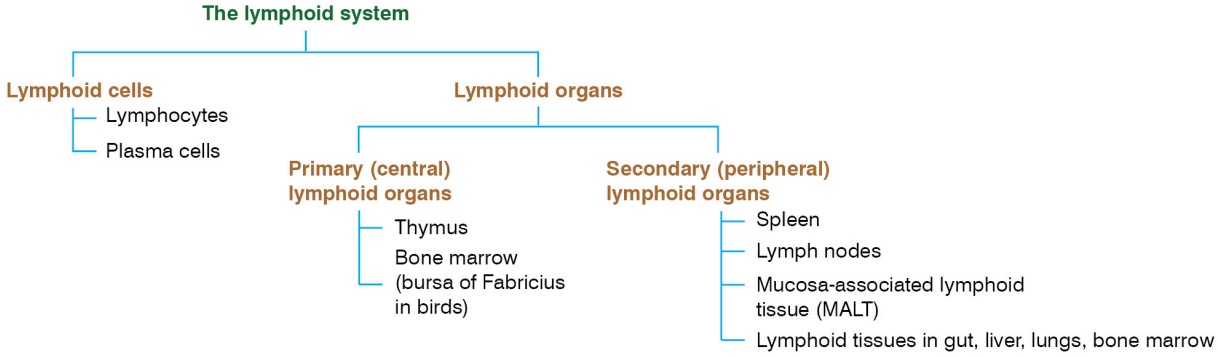


Fig. 8.3 The lymphoid system

A. CENTRAL (PRIMARY) LYMPHOID ORGANS

1. Thymus

The thymus performs the important function of generating and selecting a repertoire of T cells that are responsible for immunity. **In humans, the thymus reaches its maximal relative size just before birth. It continues to grow until about the twelfth year. After puberty, it undergoes spontaneous progressive involution, indicating that it functions best in early life.**

Functions

The primary function of the thymus is the production of thymic lymphocytes. It is the main site for lymphocyte proliferation in the body. Lymphocytes conditioned in the thymus are called **thymus-dependent lymphocytes** or **T cells**. T cells that cannot recognise the antigen–MHC complexes and those that react with the self-antigen–MHC complex (that may cause autoimmune disease) are destroyed. More than 95 per cent of all thymocytes die by apoptosis.

Clinical significance of thymus dysfunction

- **DiGeorge syndrome** Deficient cell-mediated immunity (CMI) is seen in congenital aplasia of the thymus in human beings (DiGeorge syndrome) and in mice ('nude mice').

- **Runt disease** Deficiency of CMI is evident from lymphopenia, deficient graft rejection and the so-called 'runt disease' seen in neonatally thymectomised mice.
- **Post thymectomy** Thymectomy primarily affects the CMI. It also diminishes antibody response to many types of antigens (thymus-dependent antigens) such as sheep erythrocytes and bovine serum albumin. Humoral response to other antigens is unaffected.

2. Bone Marrow

In humans and other mammals, the bone marrow is the site of B cell origin and development.

All lymphocytes originate in the bone marrow. While T lymphocytes mature in the thymus, B lymphocytes mature in the bone marrow itself. Immature B cells originating from lymphoid progenitors proliferate and differentiate within the bone marrow with the help of cytokines. **In birds, the bursa of Fabricius is the primary site for B cell maturation, equivalent to the bone marrow. A selection process within the bone marrow eliminates B cells with self-reactive antibody receptors, as in thymic selection during T cell maturation.**

In the human fetus, Peyer's patches, isolated lymphoid follicles [gut-associated lymphoid tissue (GALT)], develop and appear along with cells in the spleen and lymph nodes by the 20th week of gestation. From then on, the fetus is able to produce IgM and IgD. However, maternal IgG protects the fetus by crossing the placental barrier; IgA and IgE are not present at this stage. At birth, IgM production is enhanced but the maternal IgG level falls steadily to reach minimum levels by the third month after birth. Production of IgG by the neonate then picks up to become adequate by 2–3 years. **Full immunocompetence is attained only after the first decade of life.**

Clinical significance of bone marrow dysfunction

- Depletion or suppression of bone marrow activity primarily leads to humoral immunodeficiency (hypo- or agammaglobulinemia); this leads to increased bacterial infection.
- *X*-linked (Bruton type) agammaglobulinemia is a condition in which children suffer from repeated pyogenic infections.
- Common variable immunodeficiency (IgA deficiency along with IgG2) is another defect caused by bone marrow dysfunction.
- Transient hypogammaglobulinemia occurs when maternal antibody wanes; it becomes severe in premature babies.

B. PERIPHERAL (SECONDARY) LYMPHOID ORGANS

1. Lymph Nodes

Lymph nodes are distributed along the lymphatic vessels (Fig. 8.4). They contain lymphocytes (B and T) in the cortex, dendritic macrophages, plasma cells and interdigitating dendritic cells in the medulla.

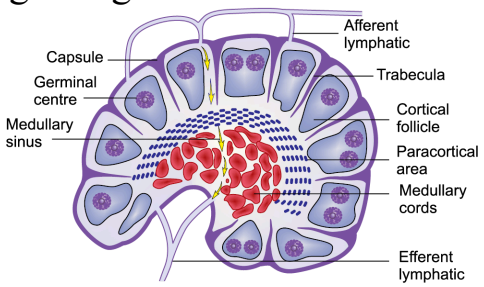


Fig. 8.4 Diagrammatic section of lymph node (arrows indicate the path of lymph flow)

Functions

Lymph nodes act as a filter for lymph, each group of nodes draining a specific part of the body. Their functions are as follows:

- Phagocytosis—foreign materials including microorganisms are phagocytosed in the lymph nodes.
- The proliferation and circulation of T and B cells

They enlarge following local antigenic stimulation (as seen in most infections, the enlargement of draining lymph nodes forms a diagnostic criterion).

2. Spleen

Spleen is the largest secondary lymphoid organ with an important role in immune response. This includes clearance of effete or damaged cells from the bloodstream and host resistance to bacterial infections, primarily directed against blood-borne antigens, mainly pneumococcal and meningococcal infections. The major anatomic compartments of the spleen are organised in such a way as to optimise the different functions of the organ. The white pulp (WP) consists of the periarteriolar lymphoid sheath (PALS), containing T cells together with the antigen-presenting cells

(APCs) that are necessary for their activation (Fig. 8.5). The PALS contains a specific type of stromal cell, the interdigitating reticulum cells (IDCs), which are characteristic of the T cell zones in the spleen.

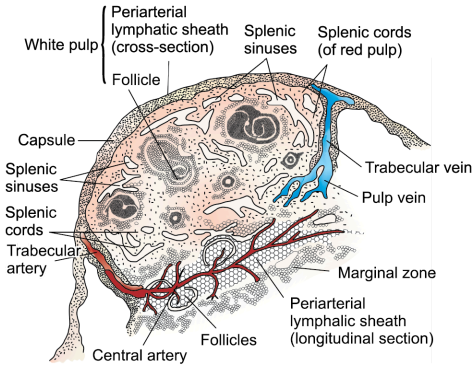


Fig. 8.5 Schematic diagram of splenic architecture

The spleen serves as:

- A graveyard for non-functional blood cells
- A reserve tank for blood
- A systemic filter for trapping circulating blood-borne foreign particles

Clinical significance of splenectomy

The effects of **splenectomy** on the immune response depend on the individual's age. In children, splenectomy often leads to increased incidence of **bacterial sepsis** caused primarily by *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*. In adults, the effects are less adverse and may lead to bacterial infections (**bacteremia**).

3. Mucosa-Associated Lymphoid Tissue (MALT)

The mucosa lining the alimentary, respiratory, genitourinary and other lumina and surfaces are constantly exposed to numerous antigens. They have a rich collection of specialised lymphoid cells like the **M cells** (**microfold cells**), which transport the antigen from the lumen of the gut to the immune cells in specialised aggregates like **Peyer's patches** or the **gut-associated lymphoid tissue (GALT)** which are scattered, isolated lymphoid follicles. **Bronchus-associated lymphoid tissue (BALT)** also is a mucosa-associated lymphoid tissue in the bronchus where the M cells take up antigens (namely, mycobacterial antigen).

MALT contains lymphoid as well as phagocytic cells. While the predominant immunoglobulin produced in the mucosa is secretory IgA, other immunoglobulin classes, IgG, IgM and IgE are also formed locally. There appears to be free traffic of antigen-specific effector lymphocytes between the various mucosal and secretory areas. Antigenic exposure at one site may cause the production of the specific antibody at the other mucosal and secretory sites. This indicates the existence of a common mucosal or secretory immune system.

Clinical significance of GALT

The existence of a common mucosal or secretory immune system may explain the superiority of oral or nasal immunisation over the parenteral route for many enteric and respiratory infections.

4. Bone Marrow

Besides being an important primary lymphoid organ, the bone marrow also has a significant role as a secondary lymphoid organ. It is capable of antigen presentation in an immunological reaction similar to the lymph nodes.

CELLS OF THE LYMPHORETICULAR SYSTEM

Lymphocytes

Lymphocytes constitute 20–40% of the body's white blood cells and 99% of the cells in the lymph. On the basis of their functions and cell-membrane components, lymphocytes can be broadly subdivided into three types:

1. T cells
2. B cells
3. Natural killer (NK) cells

Lymphocytic circulation

Constant traffic of lymphocytes through the blood, lymph, lymphatic organs and tissues ensures their contact with antigens in any part of the body. Specific lymphocytes reach the site of introduction of the antigen during their ceaseless wandering and mount an immune response. A lymphocyte completes one cycle of recirculation in about one or two days. Recirculating

lymphocytes can be recruited by the lymphoid tissues whenever necessary.

Functions

Lymphocytes are the main cellular elements responsible for an immunological response. Both T and B lymphocytes are central cells and play a vital role in generating an immune response. A lymphocyte that has been primed by the central lymphoid organs becomes an **immunologically competent cell (ICC)**. Mature T and B cells are called **naive cells** before they encounter antigens. The functions of these cells are:

- Recognition of antigens
- Storage of immunological memory
- Immune response to specific antigens

Immune response

Lymphocytes have specific antigen-recognition mechanisms on their surface. Each clone of a lymphocyte is able to recognise only one antigen. The outcome of antigen–cell interaction results in an immune response or **immune tolerance**. The nature of the immune response depends on whether the lymphocyte is a B or T cell. Stimulated T cells produce certain activation products (lymphokines) and induce CMI, while stimulated B cells divide and transform into plasma cells which synthesise immunoglobulins.

Surface markers

A number of surface antigens or markers have been identified on lymphocytes and other leucocytes with the help of monoclonal antibodies. These markers reflect the stage of differentiation and functional properties of the cells.

Order was introduced at the International Workshops for Leucocyte Differentiation Antigens by comparing the specificities of different antisera. When a cluster of monoclonal antibodies was found to react with a particular antigen, it was defined as a separate marker and given a CD (cluster of differentiation) number. Over **150 CD markers** have been identified thus far. Table 8.1 lists a few CD markers along with their cell association for CD4 (helper/inducer) and CD8 (suppressor/cytotoxic) cells.

Table 8.1 Some CD markers and their cell type association

CD number	Cell type association

CD1	Thymocytes, Langerhans cells
CD2	T cell SRBC receptor
CD3	T cell antigen–receptor complex
CD4	Helper T cell (receptor for HIV)
CD8	Suppressor/Cytotoxic T cells
CD19	B cells

Difference between T and B cells

The most clear-cut differentiation between T and B cells can be made by their surface markers—CD3 on T cells and Ig on B cells. Many other tests help in their differentiation (Table 8.2 and 8.3).

Table 8.2 Some distinguishing characteristics of T cells, B cells and macrophages

Property	T cell	B cell	Macrophage
CD3 receptor	+	–	–
Surface immunoglobulins	–	+	–
Receptor for *Fc* piece of IgG	–	+	–
EAC rosette formation (C3 receptor; CR2; EBV receptor)	–	+	–
SRBC rosette (CD2; measles receptor)	+	–	–
Thymus-specific antigens	+	–	–
Numerous microvilli on surface	–	+	–
Blast transformation with:			
a) Anti-CD3	+	–	–
b) Anti-Ig	–	+	–
c) PHA	+	–	–
d) Concanavalin A	+	–	–
e) Endotoxins	–	+	–
Phagocytic action	–	–	+

Adherence to glass surface	–	–	+

Table 8.3 Different characteristics of T and B cells

T cells	B cells
Bind to sheep erythrocytes, forming rosettes (SRBC or E rosette) by the CD2 antigen CR2 absent	Bind to sheep erythrocytes coated with antibody and complement, forming EAC rosettes Due to the presence of a C3 receptor (CR2) on the B cell surface, they do not bind
Possess T cell receptors (TCR) on the surface, composed of two chains of polypeptides, linked to CD3 Do not have surface Ig	Possess immunoglobulin on their surface Have 105 identical Ig molecules on their surface
Generally are free of cytoplasmic surface projections	Have an extensively filamentous surface, with numerous microvilli
Thymus-specific antigens are present	Thymus-specific antigens are absent
Undergo blast transformation on treatment with mitogens such as phytohemagglutinin (PHA) or concanavalin A (Con A)	Undergo transformation with bacterial endotoxins, *Staphylococcus aureus* (Cowan 1 strain) or EB virus

The first Ig class to appear on the B cell surface is monomeric IgM. Subsequently, other classes (IgG, IgA or IgE) appear, along with IgD. B cells have a single antigen specificity, depending on the immunoglobulin marker on the cell surface.

T cell

T cell maturation

T cell precursors from the yolk sac, fetal liver and bone marrow migrate to the thymus during the embryonic and postnatal stages. The earliest identifiable cells of T lineage are the $CD7^+$ pro-T cells, which acquire CD2 on entering the thymus. They synthesise CD3 in the cytoplasm and become pre-T cells. T cell receptor (TCR) synthesis also takes place during this time (Fig. 8.6).

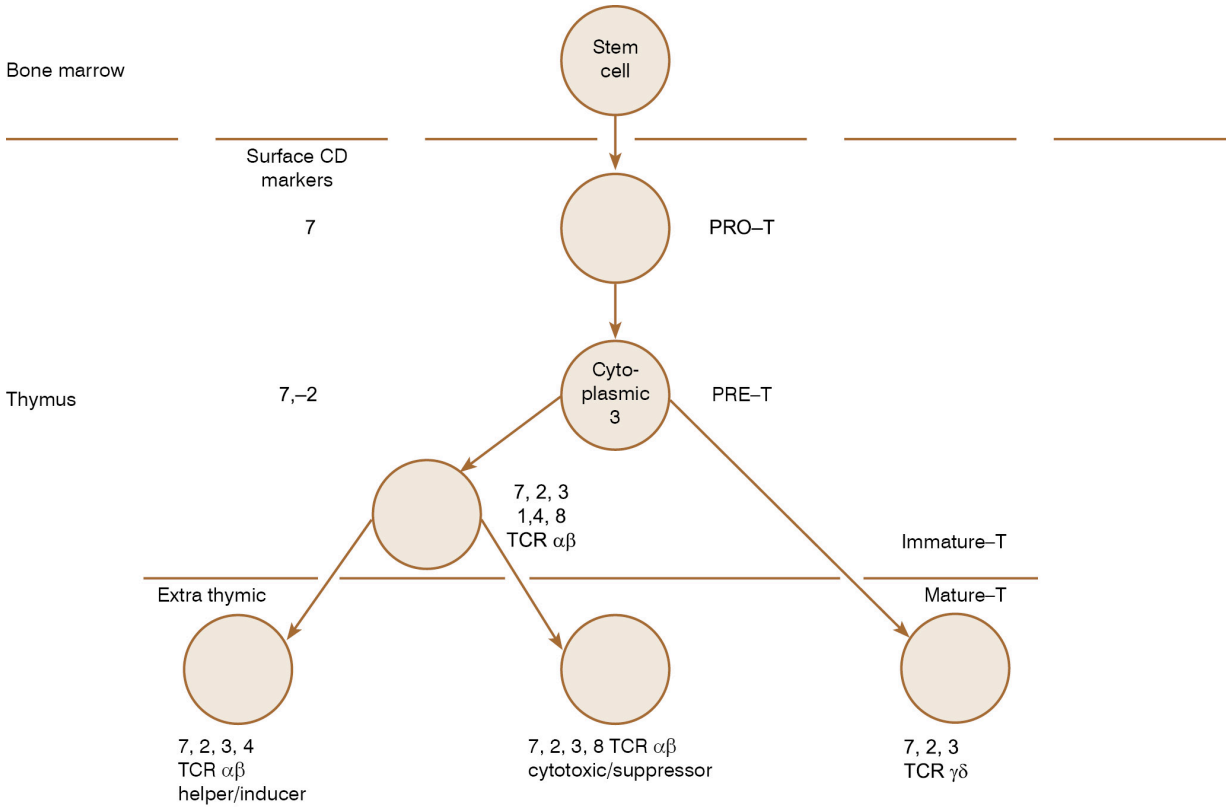


Fig. 8.6 T cell maturation

T cell receptors (TCR)

Under the influence of thymic hormones, progenitor T cells within the thymus acquire receptors called T cell receptors (TCR). T cells then undergo re-arrangement in the TCR DNA to form functional alpha (α) or beta (β) TCR complex (γ and δ may also be formed). These cells then become positive for the expression of CD4, CD8 and other co-receptors, thereby becoming functional TCR.

TCR is a heterodimer of glycoprotein chains expressed on the T cell surface, which in association with CD3, acts as the antigen recognition unit, analogous to Ig on the surface of B cells. TCR exist as two pairs of glycoprotein chains, either αβ or γδ. Pre-T cells differentiate into two lineages, expressing either αβ or γδ TCR chains. A majority of T cells carry αβ TCR (Fig. 8.7). The two chains are held together by a disulphide bond near the T cell membrane at the hinge region. TCRs have positive charges in the transmembrane portion and a short cytoplasmic tail. TCR chains contain four separately encoded regions: V or variable, D or diversity, J or joining and C or constant (**VDJC**), as in the case of immunoglobulins and

hence belong to the immunoglobulin gene superfamily. By re-assortment of these regions, a very wide repertoire of antigen specificities can be formed on the T cell surface (Fig. 8.7).

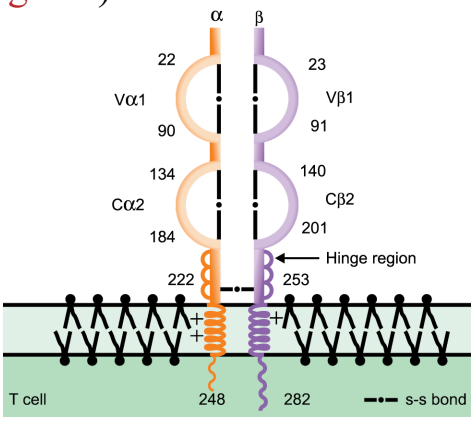


Fig. 8.7 Structure of T cell receptor (TCR)

TCR are capable of recognising host-specific antigen–MHC complexes. Contact with self-antigens within the thymus leads to the destruction of immature T cells carrying the corresponding TCR. Cells capable of reacting with autoantigens continue to arise throughout life. These potentially harmful **forbidden clones** are deleted by antigen-specific suppressor cells. Immunocompetence against foreign antigens is also developed in the thymus.

T cells also develop MHC restriction so that $CD8^+$ cells respond only to foreign antigens presented along with HLA class I and $CD4^+$ cells to those presented with HLA class II molecules. Immature T cells in the thymus exhibit CD7, 2, 3, 1, 4 and 8, besides TCR. On functional maturity, they lose CD1 and differentiate into the two major subsets: $CD8^-4^+$ or $CD4^-8^+$.

Mature $CD8^-4^+$ TCR αβ cells are helper/inducer cells. Their functions are:

- Induction of B cell differentiation
- Stimulating proliferation of $CD8^+$ cytotoxic cells
- Production of lymphokines
- Regulating certain stages of erythropoiesis

$CD4^-8^+$ TCR αβ cells are suppressor/T cytotoxic cells (T_C) whose functions are:

- Inhibiting B cell antibody synthesis
- Acting as cytotoxic effector cells

T_C cells are activated when they interact with an antigen–class I MHC complex on the surface of an altered self-cell (e.g., a virus-infected cell or a tumour cell) in the presence of appropriate cytokines. This activation results in proliferation, which causes the T_C cell to differentiate into an effector cell called **cytotoxic T lymphocyte (CTL)**, and thereby acquire the ability to recognise and eliminate altered self-cells. Small numbers of $CD4^+8^+$ and $CD4^-8^-$ cells are also present in circulation.

The function of TCR γδ cells is not well understood but they are believed to be immune surveillance cells on epithelial surfaces and a form of defence against intracellular bacteria. They are believed to participate in innate immunity and immune response homeostasis.

Antigenic changes characterising T cell maturation enable their identification. This is applied in defining T cell malignancies. Acute T cell malignancies such as lymphoblastic leukemia and lymphomas involve early T cells, pro-T cells and other immature forms. Chronic T cell malignancies like mycosis fungoides, peripheral T cell lymphomas and HTLV-1-associated adult T cell leukemias involve mature T cells, mainly $CD4^+$ cells.

Types of T cells

There are various T cell categories that have been identified based on:

- their surface markers,
- their target cells and
- their functions.

Minor subsets of $CD4^+$ cells and $CD8^+$ cells exist.

Double-negative and double-positive T cells Some T lymphocytes, while bearing the T cell receptor (TCR) may lack CD4, CD8, and markers of natural killer (NK) cells. These cells are known as **double-negative (DN) T cells** and have been described in both human and mouse models. On the other hand, some T cells rearrange their TCR beta chain to produce a pre-TCR, which complexes with CD3 molecules. These complexes lead to survival proliferation and further differentiation by upregulating expression of CD8 and CD4. These cells are known as **double-positive (DP) T cells.**

Figure 8.8 shows the major and minor subsets of T cells along with their functions.

T cells are classified functionally as **regulatory** or **effector** cells. They may have $CD4^+$ or $CD8^+$ on their surface.

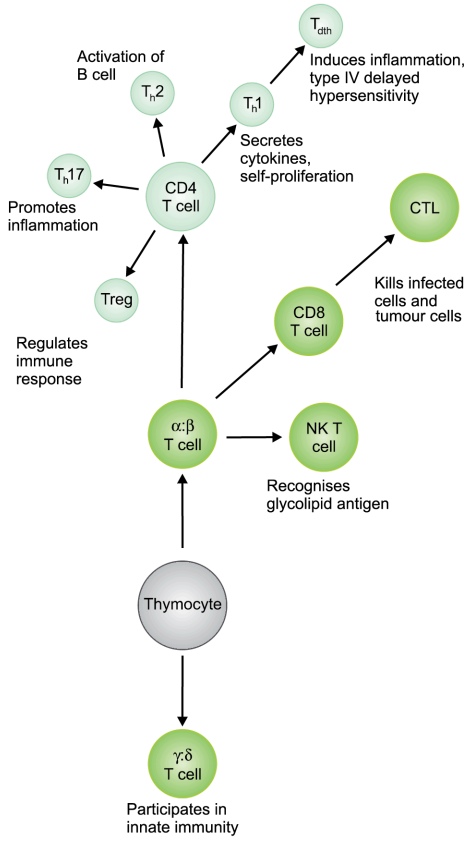


Fig. 8.8 Lineage and types of T cells and their functions

Effector T cells

Effector T cell functions include the interaction of an activated effector T cell with a target cell exhibiting a specific antigen. The effector T cell releases proteins which recognise the antigens on the target cell. The different types of effector T cells are specialised to deal with different types of pathogens, and the effector molecules are programmed to produce distinct and appropriate effects on the target cell.

- **Helper/Inducer (T_h) cells**, with **CD4 surface markers and MHC class II restriction,** generally stimulate and promote the growth of T cells and macrophages. $CD4^+$ cells can differentiate

into T_h cells or T regulatory cells. T_h cells are further differentiated into:

1. T_h1 cells, which produce cytokines, interferon gamma and interleukin-2 (IL-2), which activate macrophage and T cells, promoting cell-mediated immunity, destruction of target cells and killing of intracellular pathogens (e.g., tubercle and lepra bacilli).
2. T_h2 cells, which produce cytokines IL4, 5 and 6, which in turn stimulate B cells to form antibodies.
3. T_h17 cells, which produce cytokine IL-17 and promote inflammation [e.g., autoimmune diseases, systemic lupus erythematosus (SLE) and rheumatic arthritis] and cancer.

- **Cytotoxic/Cytolytic T cells (T_c)**, with **CD8 surface markers and MHC class I restriction**, can kill and lyse target cells carrying new or foreign antigens, including tumour, allograft and virus-infected cells.
- **Memory cells (T_m)** Another type of functional T cell is the memory T cell. Both CD4 and CD8 provide memory and anamnestic immune response. These are antigen-specific T cells that remain for a long time after an infection has been eliminated. As they are pre-stimulated to recognise the specific antigen, they quickly respond to a second stimulation by the same antigen and are converted to effector T cells. Subtypes of memory T cells are central memory T cells and effector memory T cells.

Regulatory T cells

T reg cells are $CD4^+$ cells which produce cytokine TGFβ. These cells regulate the immune response and tolerance to self-reacting cells.

B cell

B cell maturation

B lymphocyte precursors, pro-B cells, develop in the fetal liver during embryonic life and in the bone marrow afterwards and thereafter,

continuously throughout life. Rearrangement of immunoglobulin (Ig) genes takes place on pre-B cells, which synthesise cytoplasmic IgM. In the next stage—immature B cell stage—IgM is expressed on the cell surface. These cells migrate to the periphery and undergo immunoglobulin isotype switching so that instead of IgM alone, the cell expresses on its surface IgD as well as on one of the other Ig classes—IgM, IgG, IgA or IgE. **By re-assortment of Ig genes, B cells develop the capacity to produce Ig molecules which can react with all possible epitopes** (Fig. 8.9)

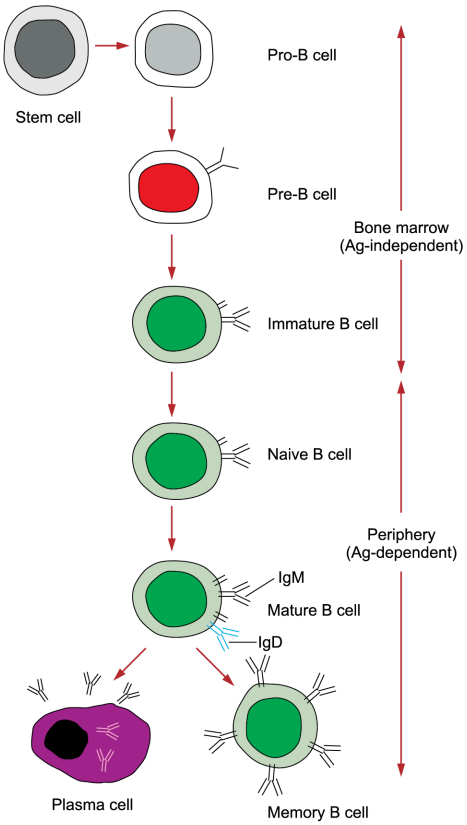


Fig. 8.9 B cell maturation

By a process of allelic exclusion, each B cell is programmed to form only one class of Ig, with either **kappa** and **lambda light chain**, possessing specificity to a single epitope alone, and expressing it on its cell surface. By contact with self-antigens during development, self-tolerance is developed by clonal deletion, leading to anergy or absence of response to antigens.

Types of B cells

On coming into contact with its appropriate antigen, the mature B cell undergoes clonal proliferation. Some activated B cells become long-living

memory cells responsible for the recall phenomenon seen on subsequent contact with the same antigen. The majority of activated B cells are transformed into plasma cells.

- ***Plasma cell*** It is the antibody-secreting cell. It is structurally designed to be an immunoglobulin-producing factory. Plasma cells are end cells and have a short lifespan of two or three days.

  A plasma cell makes an antibody of a single specificity, of a single immunoglobulin class and allotype, and of a single light chain type only. An exception is seen in the primary antibody response, when a plasma cell producing IgM initially may later switch to IgG production.
- ***Memory B cells*** Some B cells differentiate into plasma cells and others become memory B cells. These express all isotypes—IgM, IgD, IgG, IgA, and IgE— as compared to naive B cells which express only IgM and IgD. They are long-living and readily stimulated to mediate secondary immune response to subsequent encounters with the same antigen.
- ***B1 cells*** A separate lineage of B cells, predominant in fetal and early neonatal life, express the T cell marker CD5 on their surface and have been named B1 cells. Their progenitor cells move from the fetal liver to the peritoneal cavity, where they multiply. They secrete low-affinity polyreactive IgM antibodies, many of them autoantibodies. These are responsible for T-independent 'natural' IgM antibacterial antibodies which appear in neonates seemingly without antigenic stimulus. **$CD5^+$ B cells may be relevant in the causation of autoimmune conditions.**

Null cells

About 5–10 per cent of circulating lymphocytes are found to lack features of T and B cells. These are called **null cells**. Because of their morphology, they are also known as **large granular lymphocytes (LGL)**. They are nearly double the size of the small lymphocytes. They have indented nuclei and abundant cytoplasm containing several azurophilic granules, composed of mitochondria, ribosomes, endoplasmic reticulum and Golgi apparatus.

LGL are a heterogeneous group of cells with different surface markers and functional characteristics.

- **Natural killer cells (NK cells)** The most important member of this group is the natural killer (NK) cell. **The term NK cell is sometimes used as a common name for all null cells.**

  Natural killer cells possess spontaneous cytotoxicity towards various target cells, mainly malignant and virus-infected cells. Their cytotoxicity is not antibody-dependent or MHC-restricted. **NK activity is 'natural' or 'non-immune' as it does not require sensitisation by prior antigenic contact.** They are considered to be important in immune surveillance and a natural defence against virus-infected and malignant mutant cells.
  - NK cells form part of the innate immune set-up.
  - They belong to a different lineage than T and B cells.
  - They are normally active in severe combined immunodeficiency diseases (SCID) in which mature T and B cells are absent.
  - They have CD16 and CD56 on their surface.
  - They bind to the glycoprotein receptors on the surface of autologous as well as allogeneic target cells and release several cytolytic factors.
  - Cytolytic factor **perforin** resembles complement component C9, and produces transmembrane pores through which cytotoxic factors, such as the tumour necrosis factor beta (TNFβ), enter the cell and destroy it by **apoptosis** (programmed cell death).
  - NK cell activity is augmented by interferons.
- ***Antibody-dependent cytotoxic cells (ADCC)*** These are a subpopulation of LGLs possessing surface receptors for the *Fc* part of Ig. They are capable of lysing or killing target cells sensitised with IgG antibodies. This antibody-dependent cellular cytotoxicity is distinct from the action of cytotoxic T cells, which are independent of the antibody.
- ***Lymphokine-activated killer (LAK) cells*** These are NK lymphocytes treated with IL-2, which are cytotoxic to a wide

range of tumour cells without affecting normal cells. LAK cells have shown promise in the treatment of some tumours such as renal cell carcinoma. IL-2 also acts as a growth factor for NK cells.

Phagocytic Cells

These cells are specialised in the removal of foreign and native particles. Phagocytic cells comprise mononuclear macrophages (of blood and tissues) (Fig. 8.10) and polymorphonuclear microphages.

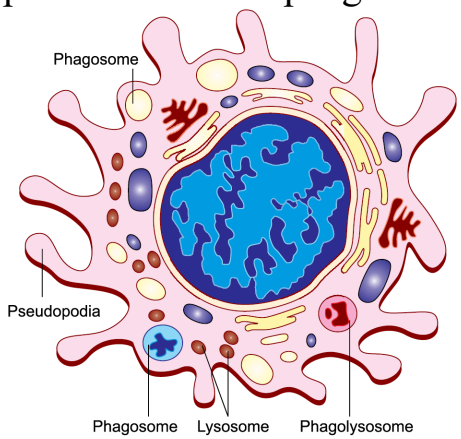


Fig. 8.10 Macrophage with phagosomes, pseudopodia and lysosome granules

1. Macrophages

They may be circulating or fixed cells.

- **Monocytes** The blood macrophages (monocytes) are the largest of the lymphoid cells found in peripheral blood (12–15 μm). Monocytes in circulation have an approximate half-life of three days. They leave the circulation and reach various tissues to be transformed into fixed macrophages.
- **Tissue macrophages** The tissue macrophages (histiocytes) are larger (15–20 μm). They have morphological and functional features characteristic of the tissues and survive for months. These macrophages have a different function to perform and are named according to tissue location as **alveolar macrophages, histiocytes, Kupffer cells, osteoclasts** and **mesangial cells**.

Clinical significance of phagocytic cells

While phagocytosis is an effective defence against most microorganisms, some (e.g., *Salmonella*, *Brucella* and mycobacteria) resist digestion and may multiply in the cells and be transported in them to other locations.

Macrophages express several surface receptors including:
i) Ia proteins,
ii) those for the *Fc* part of IgG,
iii) activated complement components and
iv) various lymphokines.

Mac 1 is a protein antigen found on mouse macrophages. A similar protein on human macrophages has been named the **M1 marker**. This appears closely related to CR3, a cell receptor for C3 components.

Mechanism of action

Macrophages participate in several ways in the induction and execution of a specific immune response. As **antigen-processing cells,** they trap the antigens, process them and present them in optimal concentration to the lymphocytes and for induction of antibodies. Too high a concentration of antigen may be tolerogenic, and too low may not be immunogenic.

Features of activated macrophages

- The functional efficiency of macrophages can be increased in many ways. They may be 'activated' by lymphokines, complement components or interferons.
- Activated macrophages are not antigen-specific. For example, activated macrophages from animals infected with one microorganism are cytotoxic to tumour cells as well as to many other microorganisms.
- Activated macrophages show morphological and functional changes as compared to unstimulated quiescent macrophages. They are larger, adhere better, spread faster on glass and are more phagocytic.
- They secrete a number of biologically active substances, including hydrolytic enzymes, binding proteins (fibronectin, transferrin), tumour necrosis factor (cachectin), colony stimulating factor (CSF) and IL-1 (formerly called the leucocyte-activating factor). IL-1 acts as an endogenous pyrogen and also induces the synthesis of IL-2 by T cells. IL-2 facilitates the activation of T cells.
- When stimulated by cytophilic antibodies and certain lymphokines, macrophages become 'armed'. Such armed macrophages are capable of antigen-specific cytotoxicity, which is important in antitumour activity and graft rejection.

2. Microphages

These are the polymorphonuclear leucocytes of the blood—neutrophils, eosinophils and basophils.

- **Neutrophils** are non-specific active phagocytes and form the predominant cell type in acute inflammation. They are augmented by opsonins. They do not have any role in specific immune processes.
- **Eosinophilic leucocytes** are found in large numbers in allergic inflammation, parasitic infection and around antigen–antibody complexes. They primarily inhabit tissues rather than the bloodstream. They have granules containing a variety of hydrolytic enzymes that lead to extracellular killing of large parasites. Eosinophils possess phagocytic activity but only to a limited degree.
- **Basophils** are found in the blood and tissues **(mast cells)**. Their cytoplasm has large numbers of prominent basophilic granules containing heparin, histamine, serotonin and other hydrolytic enzymes. Degranulation of mast cells, with release of pharmacologically active agents, constitutes the effector mechanism in anaphylactic and atopic allergy.

3. Antigen-presenting cells

Activation of both the humoral and cell-mediated branches of the immune system requires cytokines produced by T_h cells. T helper cells can recognise only antigens that are displayed together with class MHC II molecules on the surface of **antigen-presenting cells** (APCs). These are specialised dendritic cells, macrophages and B lymphocytes.

- **Dendritic cells** are the **major antigen-presenting cells** involved in the presentation of antigens to T cells during the primary immune response. They are bone marrow-derived cells of a lineage different from the macrophages and T or B lymphocytes. They possess MHC class II expression along with co-stimulatory signals like B7 and CD28 which are necessary for T_H activation. These cells are called **professional antigen-presenting cells**. They are highly pleomorphic, with a small central body and many long needle-like processes (Fig. 8.11) and are present in peripheral blood and in the peripheral lymphoid organs,

particularly in the germinal areas of the spleen, lymph nodes and skin.

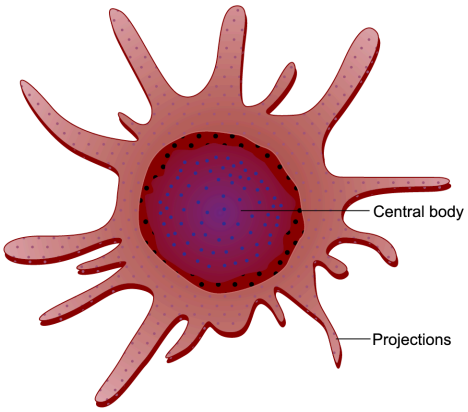


Fig. 8.11 Dendritic cell with needle-like projections

- **Follicular dendritic cells (FDCs)** are present in follicles of any secondary lymphoid organ and belong to the stromal cells of these organs. They have important functions in the selection of memory B lymphocytes during germinal centre reactions (GCR). They present native antigens to potential memory cells, of which only B cells with high-affinity B cell receptors (BCR) can bind. Ectopic FDC-formation can be found in a number of autoimmune diseases and/or chronic inflammatory situations.
- The **B cell** is another antigen-presenting cell, which is active particularly during the secondary immune response.
- **Langerhans cells** in the skin possess features of macrophages and dendritic cells. They process and present antigens that reach the dermis.

Abnormalities of Immune Cells

Abnormalities in immune cells are generally inherited defects found in either specific or non-specific immune mechanisms. Individuals suffering from such abnormalities are susceptible to a variety of infections. The type of infection depends on the nature of the cells involved and manifest as specific diseases as shown in Table 8.4.

Table 8.4 Abnormalities of the immune cells and the resulting diseases

Abnormality/Defective mechanism	Disease
Stem cell differentiation	Neutropenia: very few neutrophils
Lack of expression of CD18 molecule	LAD (leucocyte adhesion deficiency)
Defective phagocytosis	Chediak-Higashi syndrome
Defective encoding for NADPH oxidase results in defective intracellular oxygen mediated-killing	Chronic granulomatous disease leading to life-threatening bacterial and fungal infections
Defective IFN-γ IL-12 receptor	Failure to control mycobacterial infections
Congenital aplasia of the thymus	DiGeorge syndrome, deficient CMI

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

The primary function of the immune system is the recognition and elimination of foreign cells and antigens that enter the body. Tissues and organs grafted from one individual to another member of the same species (**allografts**) are recognised as foreign and rejected. It was the early work of Gorer in the 1930s on the antigens responsible for allograft rejection in inbred mice that led to the discovery of the **major histocompatibility complex** (MHC). Besides serving as cell surface markers that help infected cells to signal cytotoxic and helper T cells, the enormous polymorphism of the MHC helps maximise protection against microbial infections. By increasing the specificity of self-antigens, the MHC prevents microbes with related antigenic make-up from sneaking past host immune defences by molecular mimicry. The primary aim of the MHC may be defence against microbes and not against grafts. **It plays an important role in transplantation immunity.** The MHCs are regulated by genes**.**

MHC has been implicated in a number of non-immunological phenomena such as individual odour, body weight in mice and egg laying in chickens.

The genetic control of immune response is by specific genes called the **immune response (*Ir*) genes**. **For their work on MHC and the genetic control of immune response, Snell, Dausset and Benacerraf were awarded the Nobel Prize for Medicine in 1980.**

The name ‘histocompatibility complex’ came to be because its discovery

was based on transplantation experiments. The major antigens determining histocompatibility in human beings are alloantigens, characteristically found on the surface of leucocytes. Human MHC antigens are therefore synonymous with human leucocyte antigens (HLA), and the MHC complex of genes with the HLA complex.

Classes of Proteins

All species of animals (including human beings) are found to possess a complex of genes on a segment of one chromosome pair, coding for three different classes of proteins:

- **Class I proteins** that determine histocompatibility, and the acceptance or rejection of allografts (tissues or organs from different individuals within the same species)
- **Class II proteins** that regulate the immune response
- **Class III proteins** that include some components of the complement system and a few others

HLA Complex

The HLA complex of genes is located on the **short arm of chromosome 6** (Fig. 8.12). It consists of three separate clusters of genes:

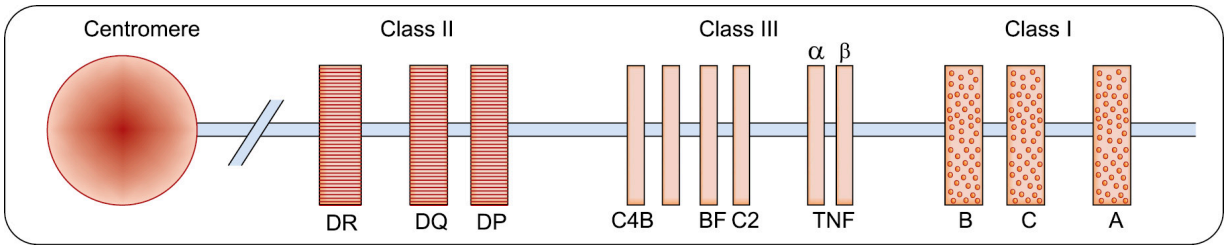


Fig. 8.12 HLA complex loci on chromosome

- **Class I**, comprising A, B and C loci
- **Class II** or the D region, consisting of the DR, DQ and DP loci
- **Class III** or the complement region, containing genes for complement components C2 and C4 of the classical pathway, as well as properdin factor B of the alternative pathway, heat shock proteins and tumour necrosis factors α and β

HLA loci are multiallelic, that is, the gene occupying the locus can be any one of several alternative forms (alleles). As each allele determines a distinct product (antigen), the HLA system is very pleomorphic. For example, at least 24 distinct alleles have been identified at HLA locus A and 50 at B.

HLA molecules

HLA antigens are two-chain glycoprotein molecules anchored on the surface membrane of cells (Fig. 8.13).

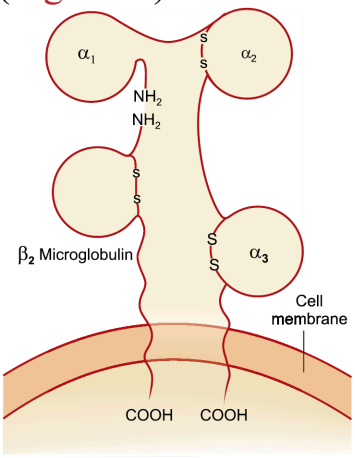


Fig. 8.13 HLA class I molecule

HLA class I molecules consist of a heavy peptide chain (alpha chain) non-covalently linked to a much smaller peptide called beta 2-microglobulin (beta chain). The beta chain has a constant amino acid sequence and is coded for by a gene on chromosome 15. The alpha chain consists of three globoid domains (alpha 1, alpha 2 and alpha 3), which protrude from the cell membrane and a small length of transmembrane C terminus reaching into the cytoplasm. The distal domains (alpha 1 and alpha 2) have highly variable amino acid sequences and are folded to form a cavity or groove between them. Protein antigens processed by macrophages or dendritic cells to form small peptides are bound to this groove for presentation to CD8 T cells. The T cell will recognise the antigen only when it is presented as a complex with the MHC class I molecule and not otherwise (MHC restriction). When so presented, the CD8 cytotoxic killer cell destroys the target cell (for example, a virus-infected cell).

HLA class I antigens (A, B and C) are found on the surface of virtually all nucleated cells. They are the principal antigens involved in

graft rejection and cell-mediated cytolysis. Class I molecules may function as components of hormone receptors.

HLA class II antigens are more restricted in distribution, being found only on cells of the immune system: macrophages, dendritic cells, activated T cells, and particularly, B cells.

Class II antigens are heterodimers, consisting of an alpha and a beta chain (Fig. 8.14). Each chain has two domains, the proximal domain being the constant region and the distal, the variable. The two distal domains (alpha 1 and beta 1) constitute the antigen-binding site for recognition by CD4 lymphocytes in a fashion similar to the recognition of the class I antigen–peptide complex by CD8 T cells. HLA class II molecules (Table 8.5) are primarily responsible for the graft-versus-host response and the mixed leuckocyte reaction (MLR).

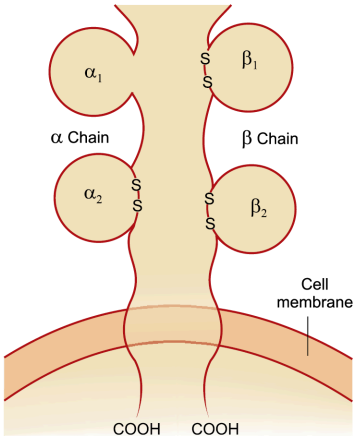


Fig. 8.14 HLA class II molecule

Both class I and II molecules are members of the immunoglobulin gene superfamily. The immune response (*Ir*) genes which control immunological responses to specific antigens are believed to be situated in the HLA class II region, probably associated with the DR locus. *Ir* genes have been studied extensively in mice and are located in the I region of mouse MHC. They code for Ia (I region-associated) antigens consisting of 1A and1E proteins.

However, the relevance of the *Ir* genes in humans is not clear.

Table 8.5 Important characteristics of HLA class I and HLA class II molecules

HLA class I	HLA class II
HLA class I antigens (A, B and C) are	Found only on cells of the immune system:

found on the surface of virtually all nucleated cells	macrophages, dendritic cells, activated T cells, and particularly B cells
They are coded for by a gene on chromosome 15	Coded on the short arm of chromosome 6
• Heavy peptide chain (alpha chain) • Beta 2-microglobulin (beta chain)	Heterodimers with an alpha and a beta chain
Highly variable amino acid sequences	Large nested sets of peptides of variable length
They are the principal antigens involved in graft rejection and cell-mediated cytolysis	These take part in graft-versus-host response and the mixed leucocyte reaction (MLR)

HLA class III molecules are heterogeneous. They include complement components linked to the formation of C3 convertases, heat shock proteins and tumour necrosis factors. They also display polymorphism.

MHC restriction Processing and presentation of an antigen by the macrophage to T cells requires that both the cells possess surface determinants coded for by the same MHC genes. The T cell can accept the processed antigen only if it is presented by a macrophage carrying on its surface the self-MHC antigens. When the macrophage bears a different MHC antigen, it cannot cooperate with the T cells. T cells respond to processed antigens on macrophages and other accessory cells only when they are presented along with the self-MHC antigen. This is known as MHC restriction. Both class I and class II antigens are involved in this phenomenon. Cytotoxic T cells can recognise class I MHC antigens on the target cells. Helper T cells can accept antigens presented by macrophages/dendritic cells only when they bear the same class II MHC molecules on the surface.

HLA Typing

Monoclonal antibodies to HLA antigens are used in microcytotoxicity, which tests for complement-mediated lysis of peripheral blood lymphocytes with a standard set of typing sera. However, serological typing is not possible for HLA-DR antigens, which are detected by the **mixed leucocyte reaction (MLR)** and **primed lymphocyte typing (PLT)**. Genetic methods are being used increasingly for HLA typing in advanced centres. These employ restriction fragment length polymorphism (RFLP) and gene

sequence-specific oligonucleotide probe typing.

The HLA antigens coded for by the combination of alleles at each locus on one strand of a chromosome pair represent the haplotype. The complete HLA type of an individual comprises the antigens represented on both strands of the diploid chromosome and so will consist of two haplotypes (e.g., HLA-A1, -A2; -B7, -B12; -Cw3, -Dw8; Dw4; -Dw7; -DR1; -DR7; DQw1; -Qw3; -DPw4; -DPw6).

Applications of HLA typing

Due to the extreme pleomorphism of the HLA system, delineation of the HLA type provides a method of typing of individuals that is far more discriminating than blood grouping.

- Transplantation HLA typing is used primarily for testing compatibility between recipients and potential donors before tissue transplantation.
- Paternity It also has applications in cases of disputed paternity.
- Anthropological studies As the prevalence of HLA types varies widely between different human races and ethnic groups, HLA typing is used in anthropological studies. Population studies of HLA polymorphism suggest the origin of the human species in Africa and emigration as different subtypes to other continents.
- Genetic predisposition to disease An association has been observed between HLA types and certain diseases. Strong association has been found between ankylosing spondylitis and HLA-B27, rheumatoid arthritis and HLA-DR4, and many autoimmune conditions and HLA-DR3.

In view of the great importance of MHC restriction in immunological control, the Nobel Prize for Medicine for the year 1996 was awarded to Peter Doherty and Rolf Zinkernagel for their seminal work on how virus-infected cells are recognised by the immune system and the specificity of the cell-mediated immune defence.

Key points

- The lymphoid system consists of the lymphoid cells and the lymphoid organs:
  - Central (primary) lymphoid organs include the thymus and the bone marrow in humans. All lymphocytes originate in the bone marrow, with T lymphocytes undergoing further differentiation in the thymus and B lymphocytes in the bone marrow.
  - Peripheral (secondary) lymphoid organs include the spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT).

- A lymphocyte that has been primed by the central lymphoid organs becomes an immunocompetent cell. Before they encounter antigens, mature T and B cells are called ‘naïve cells’, which subserve functions such as recognition of antigen and storage of immunological memory.
- Lymphocytes have antigen recognition mechanisms on their surfaces. When an immunocompetent cell reacts with its specific antigen, there may be induction of ‘tolerance’ or an immune response.
- Stimulated T cells produce lymphokines and affect cell-mediated immunity.
- A number of surface antigens or markers on lymphocytes reflect the stage of differentiation and functional properties of the cells. One such marker is the cluster of differentiation (CD) marker (CD4 in helper/inducer T cells, CD8 in suppressor/cytotoxic T cells). All T cells possess CD3 on their surface while B cells have Ig on their surface.
- T cells are classified as regulatory or effector cells. They have CD4+ or CD8+ markers on their surface.
    - Helper/Inducer (T_h) cells, with a CD4 surface marker and major histocompatibility complex (MHC) class II restriction, stimulate the growth of T cells and macrophages and can differentiate into T_h cells or T regulatory cells. T_h cells are further differentiated into T_h1, T_h2, T_h17 and Treg cells.
    - Cytotoxic T cells (T_c), with CD8 surface markers and MHC class I restriction, which can kill and lyse target cells including tumour, allograft and virus-infected cells.
    - Memory T cells (T_m), both CD4 and CD8 cells, provide memory and anamnestic immune response.
- B cells undergo maturation in different stages as pro-, pre-, immature and mature B cells.
- On contact with its specific antigen, the mature B cell undergoes clonal proliferation to form long-lived memory cells or plasma cells. These are antibody secreting cells, which makes an antibody of a single specificity, of a single immunoglobulin class and allotype and of a single light chain type only.
- Null cells are circulating lymphocytes which possess neither T cell nor B cell markers. They are of three types:
    - NK: Natural killer cells
    - ADCC: Antibody-dependent cytotoxic cells
    - LAK: Lymphokine-activated killer cells
- Phagocytic cells consist of mononuclear macrophages (of blood and tissues) and polymorphonuclear microphages and function as an effective defence against most microorganisms.
- Macrophages, dendritic cells and B cells are the major antigen-presenting cells.
- Histocompatibility antigens are cell surface antigens that induce an immune response leading to the rejection of allografts coding for three different classes of proteins:
    - Class I proteins that determine histocompatibility and acceptance or rejection of allografts

    - Class II proteins that regulate the immune response
    - Class III proteins that include some components of the complement system
- Human major histocompatibility complex (MHC) antigens are synonymous with human leucocyte antigens (HLA) and the MHC complex of genes with the HLA complex. The HLA complex of genes consists of three separate clusters of genes:
    - HLA class I, comprising A, B and C loci; found on the surface of virtually all nucleated cells, involved in graft rejection and cell-mediated cytolysis
    - HLA class II or the D region, consisting of DR, DQ and DP loci; found only on cells of the immune system (macrophages, dendritic cells, activated T cells, and particularly on B cells)
    - HLA class III or the complement region, containing genes for complement components C2 and C4; they are heterogenous
- The components of the MHC system, in the natural state, serve as cell markers that help infected cells to signal cytotoxic and helper
- T cells. The enormous polymorphism of MHC helps maximise protection against microbial infections.
- T cells respond to processed antigens on the macrophages and other accessory cells only when they are presented along with the self-MHC antigen—the phenomenon of MHC restriction.

Questions

Short Answers

1. Differences between B and T cells and their process of maturation
2. Types of T cells and their functions
3. Classes of major histocompatibility complex (MHC) and their role in immune cells
4. HLA, its classes and their functions

Part II: Immunology

Chapter 9

Antigens

Chapter navigation

- Antigen recognition molecules
- Antigen classes
- Antigen processing

INTRODUCTION

An **antigen,** in general, is any substance that induces an immune response in a host. It stimulates the production of an antibody in a specific and observable manner. The following are some definitions closely related to the study of antigens.

Antigen Substances that can be recognised by B cell receptors (Ig) and T cell receptors [along with the major histocompatibility complex (MHC)], and that evoke a specific immune response are called antigens. The **nature/molecular configurations and properties of an antigen as well as the immune system of the host, both play an important role in eliciting an immune response**. Both T and B lymphocytes recognise each antigen differently owing to the presence of different antigen recognition sites on each type of lymphocyte. Antigens can be:

- **Complete antigens** that are able to evoke an immune response independently.
- **Partial antigens** that require an additional carrier protein to be able to stimulate an antibody response. These are known as **haptens**. Haptens are substances that are incapable of inducing antibody formation by themselves but can react specifically with antibodies (in Greek, *haptein* means 'to fasten'). Haptens become immunogenic (capable of inducing antibodies) on combining with a protein carrier. They may be:

- **Complex haptens,** which are polyvalent and can precipitate with specific antibodies.
- **Simple haptens,** which are univalent and non-precipitating since it is assumed that precipitation requires the antigen to have two or more antibody-combining sites. They can inhibit the precipitation of specific antibodies by the corresponding antigen or complex hapten.

Antigenicity It is the property that allows a substance to combine specifically with antibodies or TCRs, whether or not they are immunogenic. An immunogen can trigger an immune response and act as an antigen in that immune response. **Thus, all immunogens are antigens but all antigens are not immunogens.**

Immunogen A substance that induces a detectable immune response (humoral or cellular) is called an immunogen. These substances stimulate the immune reaction, resulting in either the production of antibodies or the activation of T cells, finally leading to either immune response or immune tolerance.

Immunogenicity It refers to the ability to induce humoral and/or cell-mediated immune response.

Epitope The smallest unit of an antigen responsible for antigenicity is known as the **antigenic determinant** or **epitope**. The epitope consists of four or five amino acids or monosaccharide residues, possessing a specific chemical structure, electrical charge and steric (spatial) configuration. It is capable of sensitising an immunocyte and of reacting with its complementary site on the specific antibody or TCR.

Epitopes may be present as sequential or conformational epitopes.

- **Sequential or linear epitope** is a single linear segment of the primary sequence (Fig. 9.1a). T cells recognise sequential epitopes.
- **Conformational epitopes** are formed by being brought together on surface residues from different sites of the peptide chain during its folding into the tertiary structure (**epitope**) (Fig. 9.1b).

B cells identify the tertiary configuration of the conformational epitopes.

Paratope This is the area on the antibody molecule that corresponds to the epitope. The epitopes and paratopes determine the specificity of immunological reactions. Antigens such as bacteria or viruses carry many different types of epitopes, presenting an **antigenic mosaic**. The presence of the same or similar epitopes on different antigens accounts for one type of antigenic cross-reaction.

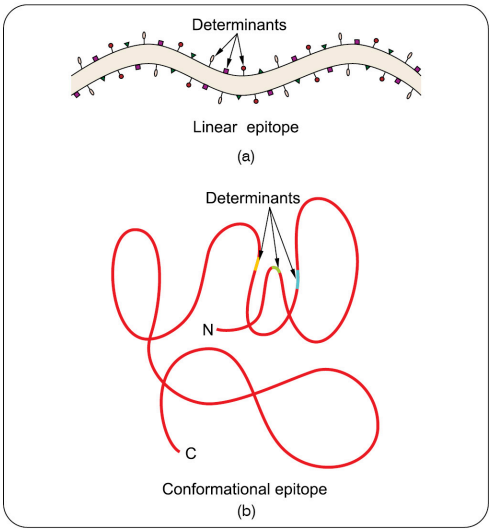


Fig. 9.1 Epitopes: (a) linear and (b) conformational

DETERMINANTS OF ANTIGENICITY

A number of properties that make a substance antigenic have been identified, but the exact basis of antigenicity is still not clear.

1. ***Foreignness*** Only antigens that are 'foreign' to the individual (non-self) induce an immune response. An individual does not normally mount an immune response against her own normal constituent antigens. This was first recognised by Ehrlich, who proposed the concept of **horror autotoxicus** (fear of self-poisoning). Tolerance of self-antigens is conditioned by contact with them during the development of the immune apparatus. Breakdown of this homeostatic mechanism results in autoimmunisation and

autoimmune disease. **Antigenicity of a substance is related to the degree of its foreignness**.

- Antigens from individuals of the same species are less antigenic than those from other species.
- Antigens from related species are less antigenic than those from distant species.

2. ***Size*** Antigenicity is related to molecular size. Large molecules are highly antigenic and particles with low molecular weight (less than 10,000) are non-antigenic or feebly so. Low-molecular-weight substances may be rendered antigenic when they combine with tissue proteins. These substances are haptens of low immunogenicity, effective in some persons only (e.g., penicillin is a low-molecular-weight antigen, which combines with tissue proteins and becomes antigenic. It can produce hypersensitivity in some persons, leading to anaphylaxis).
3. ***Chemical nature*** Most naturally occurring antigens are proteins and polysaccharides. Lipids and nucleic acids, which are less antigenic, can become more antigenic by combining with proteins, which are composed of about 20 different amino acids. They are better antigens than polysaccharides, which have only four or five monosaccharide units. However, not all proteins are antigenic.
4. ***Susceptibility to tissue enzymes*** Antigens introduced into the body are degraded by the host into fragments of appropriate size containing the antigenic determinants. Phagocytosis and intracellular enzymes appear to play an essential role in breaking down antigens into immunogenic fragments. Substances not susceptible to tissue enzymes are not antigenic.
5. ***Other factors*** Some other factors that play a role in immune response are the dosage, route and timing of the antigen administration. An antigen evokes optimum response when administered in the right quantity. An excess dose causes immunological tolerance while an inadequate dose fails to evoke adequate response. Antigens administered intravenously evoke rapid

response while the subcutaneous or intradermal route elicit only a slow release.

Adjuvant These are substances that enhance the immunogenicity of an antigen (when mixed with it). They are often used to boost the immune response when an antigen has low immunogenicity. An adjuvant stimulates the immune response by helping the antigen gain entry into the antigen-presenting cell. Some examples are:

- **Mineral salts**, e.g., aluminium hydroxide ('alum'), aluminium phosphate and calcium phosphate
- **Oil emulsions,** e.g., MF59, a detergent-stabilised oil-in-water emulsion
- **Particulate adjuvants**, e.g., virosomes and ISCOMS (structured complex of saponins and lipids)

ANTIGENIC SPECIFICITY

The basis of specificity of an antigen is its **stereochemistry** and **spatial configuration.** Antigenic specificity is not absolute. Cross-reactions can occur between antigens that bear stereochemical similarities. In some instances, apparent cross-reactions may actually be due to the sharing of identical antigenic determinants by different antigens.

Obermayer and Pick showed that antigenic specificity is determined by single chemical groupings and even by a single acid radical. The importance of the position of the antigenic determinant group in the antigen molecule was evidenced by the differences in specificity in compounds with the group attached at the ortho, meta or para positions. The influence of spatial configuration of the determinant group was shown by differences in the antigenic specificity of the dextro, levo and meso isomers of tartaric acid. This was later confirmed by Landsteiner.

Types of Specificity

The specificity of natural tissue antigens is differentiated as follows:

- ***Species specificity*** Tissues of all individuals in a species contain species-specific antigens. There exists some degree of cross-reaction between
  antigens from related species. This immunological relationship parallels their phylogenetic relationship. Species specificity has

been used in: a) tracing evolutionary relationships between species and b) forensic applications in the identification of the species from blood and seminal stains.

    - Phylogenetic relationships are reflected in the extent of cross-reactions between antigens from different species, which causes hypersensitivity. An individual sensitised to horse serum will react with serum from other equines but may not do so with bovine serum.

- ***Isospecificity*** Isoantigens are antigens found in some but not all members of a species. A species may be grouped depending on the presence of different isoantigens in its members. The best examples of isoantigens are the **human erythrocyte antigens**, based on which individuals can be classified according to the different blood groups. These are genetically determined. They are of clinical importance in **blood transfusion** and in **isoimmunisation during pregnancy**; blood groups also find application in **anthropology**. In the past, isospecificity was helpful in resolving disputed paternity cases. However, in recent times, such tests have been replaced by the more discriminatory DNA fingerprinting tests.

Histocompatibility antigens are cellular determinants that are specific to each individual of a species. They are recognised by genetically different individuals of the same species when attempts are made to transfer or transplant cellular material including organs from one individual to another.

- ***Autospecificity*** Autologous or self-antigens are ordinarily non-antigenic but there are exceptions. Sequestered antigens that are not normally found free in circulation or tissue fluids (such as the eye lens protein normally confined within its capsule) are not recognised as self-antigens. Similarly, antigens that are absent during embryonic life and develop later (such as the sperm) are also not recognised as self-antigens.
- ***Organ specificity*** Some organs, such as the brain, kidneys and the lens protein of different species share the same antigen. Such antigens, characteristic of an organ or tissue and found in different species, are called organ-specific antigens.

The neuroparalytic complications following anti-rabies vaccination using sheep brain vaccines are a consequence of brain-specific antigens shared by sheep and human beings. The sheep brain antigens induce immunological response in the vaccinees, damaging their nervous tissue. Animal-derived vaccines are no longer used in humans.

- ***Heterogenetic specificity (heterophile antigens)*** Sometimes, the same antigens or closely related antigens may occur in different biological species, classes and kingdoms. These are known as heterogenetic or heterophile antigens.

Forssman antigen

It is a heterophile antigen having a lipid–carbohydrate complex and is widely distributed in many animals, birds, plants and bacteria. It is, however, absent in rabbits. For this reason, anti-Forssman antibodies can be prepared in these animals.

Heterophile reaction

Other heterophile antigens are used for some diagnostic tests in serological reactions. Antigens unrelated to the etiological agents are employed. Some examples are:

- Weil–Felix reaction in typhus fever
- Paul–Bunnell test in infectious mononucleosis
- Cold agglutinin test in primary atypical pneumonia

BIOLOGICAL CLASSES OF ANTIGENS

Depending on their ability to induce antibody formation, antigens are classified into:

1. T cell-dependent (TD) antigens
2. T cell-independent (TI) antigens
3. Superantigens

T Cell-Dependent Antigens (TD)

Most natural proteins are T-dependent antigens. B cells cannot respond to these antigens without a co-stimulatory signal from the T_h cells. Structurally, these antigens are characterised by a few copies of many **different antigenic determinants**. These antigens bind to the surface Ig on

B cells and are internalised and processed to smaller peptides, which are then expressed on the surface of B cells complexed with MHCII and presented to T cells. Once these complexes are recognised by T_h cells, they secrete cytokines and start expressing the **CD40 ligand**, which interacts with CD40 on the B cells. **The T–B interaction and cytokines provide the stimulus for B cell activation.**

T Cell-Independent Antigens (TI)

Some antigens can **directly stimulate antibody production by B cells**, without the apparent participation of T cells. Such antigens arecalled **TI antigens**. These antigens react with B cell antigen receptors (BCRs) of innate immunity. Most microbial carbohydrates, lipids and certain nucleic acids are T cell-independent antigens. They are of two types:

- **Type 1 antigens** [endotoxin, lipopolysaccharide (LPS)] are directly mitogenic for B cells and cause **polyclonal** B cell activation.
- **Type 2 antigens** are polymeric compounds like polysaccharides (bacterial cell wall lipopolysaccharide or pneumococcal capsular polysaccharide) or proteins (flagellar proteins). They activate B cells to generate specific antibodies with the help of cytokines, complement and other cells like macrophages, dendritic cells, mast cells and NK cells.

Some of the differences between T cell-dependent and T cell-independent antigens are mentioned in Table 9.1.

Table 9.1 Comparison between T cell-dependent and T cell-independent antigens

Property	T cell-dependent antigen	T cell-independent antigen
T cell involvement	Yes	No
Antigen interaction	Involves tertiary complex of T cell receptor, Ag, MHC molecule	Involves binary complex of membrane Ig and Ag
Antigen processing by	Yes	No

macrophages		
Chemical nature of antigen	Mostly soluble protein	Type 1: LPS Type 2: polymeric proteins and polysaccharide
Degradability	Easily degradable	Type 2: poorly degradable
Complement activation	No	Type 2: Ag causes complement activation
Isotype switching	Yes	Type 1: no Type 2: limited
Immunological memory	Yes	No
Polyclonal activation	No	Type 1: yes, Ag in high doses
Effect on antibody production	Full range, IgM, IgG, IgA and IgE	Limited to IgM and IgG3

Superantigens

Superantigens are certain protein molecules (e.g., staphylococcal enterotoxins) that activate very large numbers of T cells irrespective of their antigenic specificity. Superantigens bind outside the antibody-binding groove, directly to the lateral aspect of the T cell receptor (TCR) β chain, while conventional antigen fragments bind to the αβ heterodimer groove of the MHC molecules through the V regions of the TCR α and β chains (Fig. 9.2).

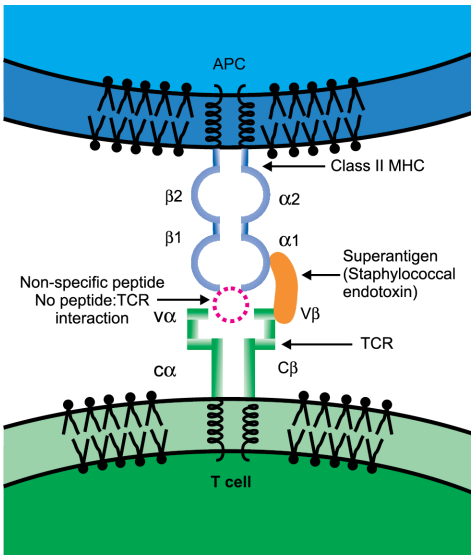


Fig. 9.2 Schematic diagram of a superantigen

Microbial superantigens are medium-sized proteins (MW 22–29 kDa) characterised by high resistance to proteases and to denaturation by $CD4^+$ T cells. They cause the release of cytokines (IL-2), which results in massive proliferation of T lymphocytes. This ultimately leads to further release of a variety of cytokines which can have profound effects on the immune system. Some human diseases associated with superantigens are presented in Table 9.2.

Table 9.2 Human diseases associated with microbial superantigens

Organism	Superantigen	Disease
Staphylococcus aureus	Enterotoxins A–E, G–J, K–R, U and V Toxic shock syndrome toxin (TSST-1, same as enterotoxin F)	Food poisoning Toxic shock syndrome Multiple sclerosis
Group A streptococci	Pyrogenic exotoxins (This is associated with streptococcal toxic shock syndrome and scarlet fever)	Shock Psoriasis Rheumatic heart disease
Mycobacterium tuberculosis	Not identified	Tuberculosis
HIV	Nef (negative regulatory factor)	AIDS
Rabies virus	Nucleocapsid protein	Rabies
Epstein–Barr virus	Not identified	B cell lymphoma Transactivates another

		endogenous virus HERV-K18
Cytomegalovirus (CMV)	Not identified	Causes enhanced HIV replication in Vβ12 T cells, which are reservoirs for HIV

DETERMINANTS RECOGNISED BY THE INNATE IMMUNE SYSTEM

The receptors of the adaptive immune system recognise discrete determinants and demonstrate a high degree of specificity, enabling this system to recognise and react to a particular pathogen. In contrast, components of the innate immune system recognise unique molecular patterns which are shared by many related pathogens but not their host. Hence, immunity has the property of pattern recognition. The broad molecular patterns are called **pathogen-associated molecular patterns** or **PAMPs** and the receptors for PAMPs are called **pattern recognition receptors** or **PRRs**. A particular PRR can recognise a molecular pattern that may be present on a number of different pathogens, thereby enabling the receptor to recognise a variety of different pathogens.

PRRs are divided into three classes:

- ***Toll-like receptors (TLRs)*** These are transmembrane receptors present on macrophages and dendritic cells. There are 13 different TLRs that recognise pathogens, enhance phagocytosis and further lead to inflammation at the site.
- ***Scavenger receptors*** These include CD36, CD68 and SRB-1. These can bind to bacterial cell wall components such as LPS, peptidoglycans as well as infected, injured or apoptotic cells and modified lipoproteins.
- ***Mannose receptors*** These receptors on the surface of phagocytes bind mannose-rich glycans, which are short carbohydrate chains with mannose, N-acetyl glucosamine or fucose as the terminal sugar. They are commonly found in microbial glycoproteins and glycolipids but are rare in those of

humans. They help to internalise the bacterial antigen into the phagocyte.

Key points

- A substance that induces an immune response is called an antigen. It stimulates the production of an antibody, with which it reacts specifically, generally in an observable manner. Antigenicity refers to the ability of an antigen to induce an immune response.
- An immunogen is a substance that can induce an immune response but which does not necessarily bind to its specific antibody.
- A hapten is a small molecule which, by itself, is not immunogenic but which can form a complex with a large molecule to induce a specific immune response.
- Most antigens are foreign to the host. They are large molecules, such as proteins and polysaccharides. Small chemical groups on the antigen molecule, called epitopes, constitute the areas that are recognised by antibodies.
- Molecular properties like size, chemical complexity and foreignness ultimately contribute to the activation of the immune system by the antigens.
- T cell-independent antigens can directly stimulate antibody production by B cells, without the apparent participation of T cells, whereas T cell-dependent antigens require a co-stimulatory signal and cytokines from T_H cells.
- Superantigens are protein molecules that bind outside the antibody-binding groove directly to the lateral aspect of the TCR β chain and activate very large numbers of T cells irrespective of their antigenic specificity.
- Toll-like receptors, scavenger receptors and mannose receptors are components of the innate immune system and recognise unique molecular patterns which are shared by many related pathogens but not with their host; hence they recognise a variety of different pathogens/antigens.

Questions

Essay

1. Define antigens. Describe the various determinants of antigenicity.

Short Answers

1. Differences between T cell-dependent and T cell-independent antigens
2. Factors responsible for the antigenicity of a molecule
3. Haptens
4. Characteristics of superantigens with examples
5. Epitopes (linear and conformational)
6. Toll-like receptors

7. Mannose receptors

Part II: Immunology

Chapter 10

Antibodies

Chapter navigation

- Antibody structure and function
- Immunoglobulin classes
- Abnormal immunoglobulins
- Immunoglobulin diversity
- Class switching
- Monoclonal antibodies

INTRODUCTION

Antibodies are proteins, collectively called immunoglobulins (glycoprotein molecules), that react specifically to an epitope on an antigen which specially stimulated their production precisely. They are present on the B cell membrane and are secreted by plasma cells. Antibodies constitute 20% of plasma proteins. They circulate in blood and eliminate or neutralise the antigen by their effector functions such as:

- Phagocytosis
- Antibody-dependent cell-mediated cytotoxicity (ADCC)
- Opsonisation

Tiselius (1937) separated serum proteins into albumin and alpha, beta and gamma globulins based on their electrophoretic mobility (Fig. 10.1). Tiselius and Kabat (1938) showed that antibody activity was associated with the gamma globulin fraction. Most antibodies are found in these gamma globulin fractions and are hence named **immunoglobulins** (Ig).

The generic term '**immunoglobulin**' was endorsed by WHO in 1964, after which it became internationally accepted for '**proteins of animal origin endowed with known antibody activity**' and for certain other proteins related to them by chemical structure. This definition includes:

- Antibody globulins
- Abnormal proteins found in myeloma
- Macroglobulinemia
- Cryoglobulinemia
- Naturally occurring subunits of immunoglobulins

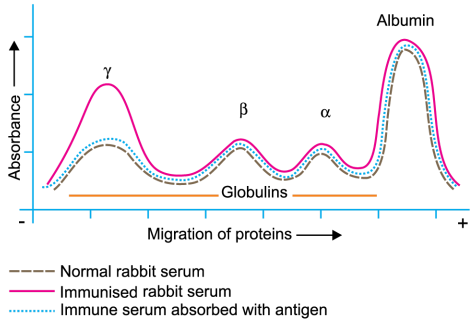


Fig. 10.1 Serum electrophoresis showing γ globulin nature of antibodies

Immunoglobulins are **structural and chemical concepts,** which are synthesised by plasma cells (and to some extent by lymphocytes). 'Antibody' **is a biological and functional model. All antibodies are immunoglobulins, but all immunoglobulins may not be antibodies.**

Immunoglobulins constitute 20–25 per cent of total serum proteins. Based on physicochemical and antigenic differences, **five classes of immunoglobulins have been recognised: IgG, IgA, IgM, IgD and IgE.**

ANTIBODY STRUCTURE

Antibody structure has been studied by cleaving the structural components of antibodies by enzymatic action. Based on these studies, it has been found that antibodies contain two identical light (L) chains of 22,000 Da and 2 similar heavy (H) chains of 55,000 Da.

Enzyme Digestion

Studies involving the cleavage of the immunoglobulin molecule were pioneered by Porter, Edelman, Nisonoff and their colleagues.

Papain digestion When rabbit IgG antibody to egg albumin was digested by papain in the presence of cysteine, it split into two fractions:

- ***Fc*** (Fragment crystallisable)—an insoluble fraction which crystallised in the cold and
- ***Fab*** (Fragment antigen binding)—a soluble fragment which was unable to precipitate with egg albumin, but could still bind with it.

Each molecule of immunoglobulin was split by papain into **three parts**, one *Fc* and two *Fab* pieces, having a sedimentation coefficient of 3.5S.

Pepsin digestion When treated with **pepsin**, a 5S fragment was obtained, which was composed essentially of **two *Fab*** fragments held together in position. This was observed to be the active part that precipitated with the antigen. This fragment was called ***F(ab)$_2$*.** The *Fc* portion can be digested by pepsin into smaller fragments (Fig. 10.2).

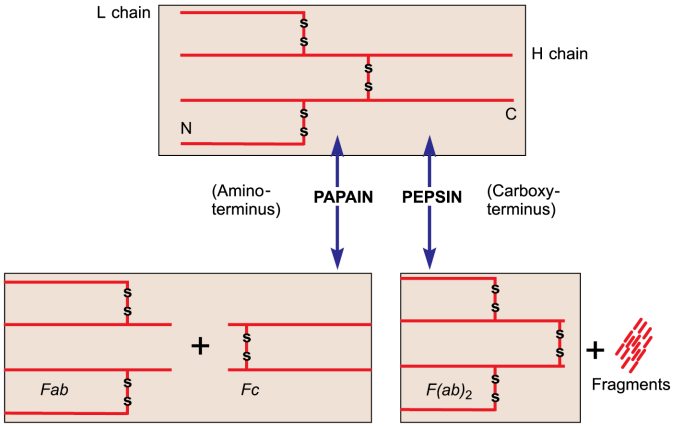


Fig. 10.2 Basic structure of an immunoglobulin molecule and the fragments obtained by cleavage by papain and pepsin

Mercaptoethanol treatment This cleaves the disulphide bonds of the antibody to its four-subunit structure (Fig. 10.3).

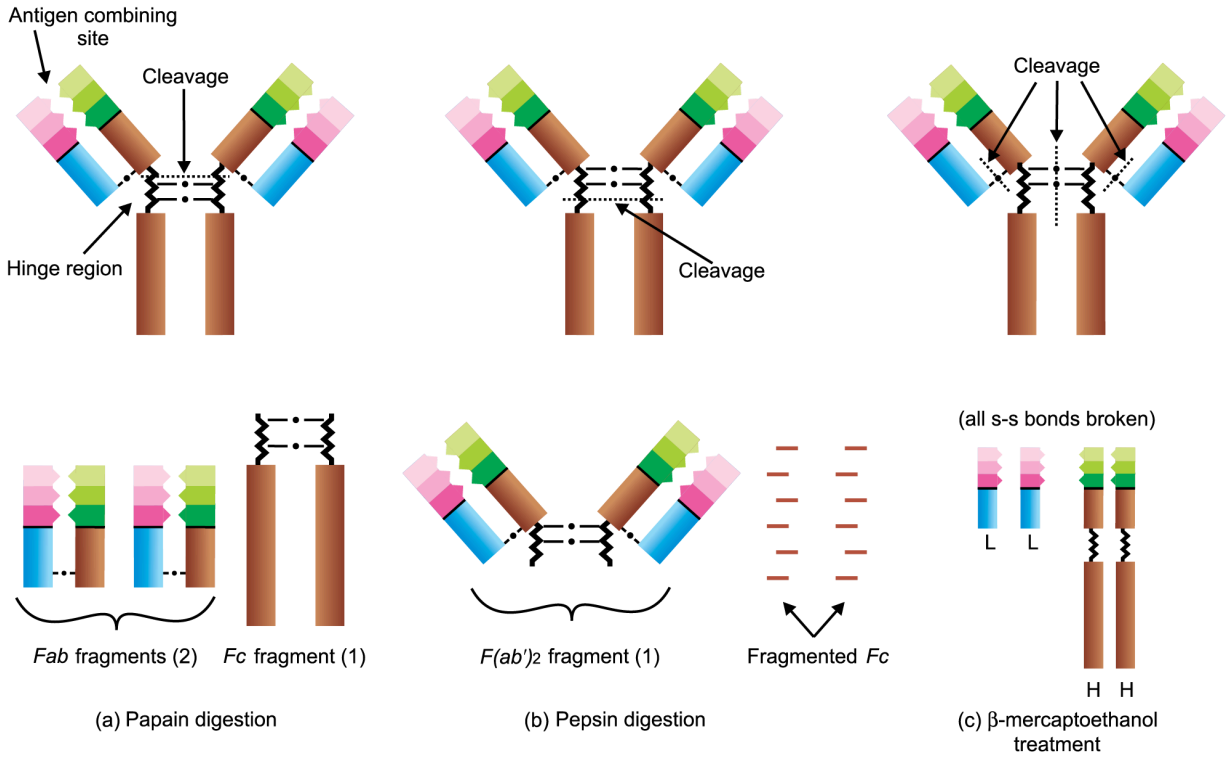


Fig. 10.3 Cleavage of an antibody molecule using: (a) papain; (b) pepsin and (c) mercaptoethanol

Immunoglobulin chains Antibodies or immunoglobulins are glycoprotein molecules consisting of 4 polypeptide chains, which are as follows:

- Two identical **heavy (large) chains (H),** molecular weight >55 kDa
- Two identical **light (small) chains (L)**, molecular weight >22 kDa

The H chains are structurally and antigenically distinct for each class and are designated by the Greek letter corresponding to the immunoglobulin class. The heavy chains are of five types—**alpha** (α), **gamma** (γ), **delta** (δ), **epsilon** (ε) and **mu** (∝) (Table 10.1).

Table 10.1 Immunoglobulin classes and H chains

Immunoglobulin class	H chain
IgG	γ (gamma)
IgA	α (alpha)

IgM	μ (mu)
IgD	δ (delta)
IgE	ε (epsilon)

The L chains are similar in all classes of immunoglobulins.

There are two types of light chains, **kappa** (κ) and **lambda** (λ). A molecule of immunoglobulin may have either kappa or lambda chains but never both together. In humans, 60 per cent of L chains are kappa and 40 per cent are lambda. Each light chain is bound to a heavy chain by interchain–intrachain disulphide bonds (Fig. 10.4).

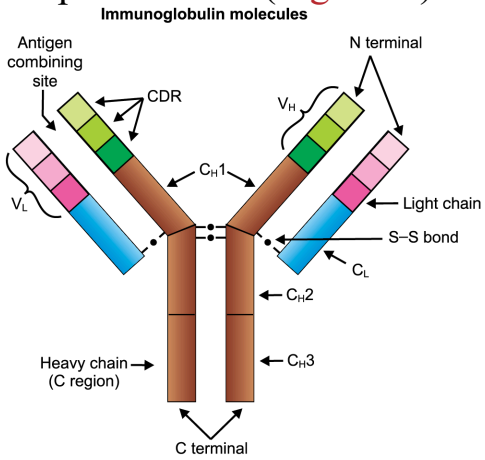


Fig. 10.4 Structure of an immunoglobulin molecule

Variable and Constant Regions

The antigen-combining site of the immunoglobulin molecule is at its **amino-terminus**. It is composed of both L and H chains. The first 110 amino acids of the N terminal are quite variable in amino acid sequence; this region is called the **variable region**. It is made up of:

- V_L, **variable light chain** and
- V_H, **variable heavy chain**.

The sequence beyond the variable region in all antibodies is relatively constant throughout the rest of the molecule and is called the **constant region (C)**. It is made up of:

- C_L, **constant light chain** and
- C_H, **constant heavy chain**.

The carbohydrate moieties are linked to the constant region of the H chains. The amino terminal variable region of light and heavy chains participates in antigen recognition and the carboxyl terminal constant region of heavy chains mediates the effector functions. The C region of the light chains does not attach to the cell membrane and does not participate in its effector functions.

Immunoglobulin Domains

The L and H chains contain several homologous units. Within each unit, an **intrachain disulphide** bond forms a loop of 60 amino acids, called the **domain**.

- The light chain contains one variable domain V_L and one constant domain C_L.
- The heavy chain contains one variable domain V_H and three or four constant domains, depending on the Ig class: C_H1, C_H2, C_H3, and C_H4.

The variable and constant domains have a similar structure, except for some subtle differences. For example, the V domain is slightly longer than the C domain; it contains an extra pair of β strands and an extra loop sequence connecting this pair of β strands. The quaternary structure of the immunoglobulin is facilitated by non-covalent interactions between domains across the faces of the β strand. Interactions occur between two non-identical domains of heavy and light chains; for example, V_H/V_L and C_H1/C_L and between identical domains as C_H2/C_H2, C_H3/C_H3 or C_H4/C_H4.

Immunoglobulin fold X-ray crystallographic analysis has revealed that Ig domains are folded into a characteristic compact structure known as the immunoglobulin fold. The structure of the Ig domain is a sandwich of two β-antiparallel pleated sheets stabilised by hydrophobic interactions between them and by disulphide bonds.

Hypervariable and Framework Regions

In the variable region of the H and L domains, maximum sequence variation is concentrated in a few discrete regions called **hypervariable (HV) regions;** the less variable stretches are termed **framework (FR) regions. HV regions form the antigen-binding site of the antibody molecule, are complementary to the structure of the epitope and are called complementarity determining regions (CDRs).** Each *Fab* fragment has six CDRs (three in H and three in L). The wide range of specificity exhibited by antibodies is a function of variations in the length and amino acid composition of the six CDRs. The framework region acts as a scaffold to support six CDR loops in each *Fab* fragment. Crystallographic studies suggest that out of the six CDRs, only four mainly make contact with the antigen's epitope.

Constant Region Domains

C region domains are associated with various biological functions determined by the amino acid sequence of each domain. The presence of C_H1 and C_L domains appears to increase the number of stable V_H and V_L interactions possible, thus contributing to the overall diversity of the antibody molecule.

Hinge Region

An extended amino acid sequence between the C_H1 and C_H2 domains is called the hinge region. It is rich in **proline** and **cysteine** amino acids and is more flexible. The number of disulphide bonds varies in different classes and subclasses of immunoglobulins. The γ, δ and α heavy chains have hinge regions but the μ and ε chains lack it; thus, they have an additional domain of 110 amino acids (C_H2/C_H2) with hinge-like features.

IMMUNOGLOBULIN CLASSES

Human sera contain the immunoglobulins IgG, IgA, IgM, IgD and IgE in a descending order of concentration. Table 10.2 lists their characteristics.

Table 10.2 Characteristics of immunoglobulin classes

Characteristic	IgG	IgA*	IgM	IgD	IgE
Sedimentation coefficient (S)	7	7	19	7	8

Molecular weight	150,000	160,000	900,000	180,000	190,000
Serum concentration (mg/mL)	12	2	1.2	0.03	0.00004
Half-life (days)	23	6	5	2–8	1–5
Daily production (mg/kg)	34	24	3.3	0.4	0.0023
Intravascular distribution (per cent)	45	42	80	75	50
Carbohydrate (per cent)	3	8	12	13	12
Complement fixation					
Classical	++	–	+++	–	–
Alternative	–	+	–	–	–
Placental transport	+	–	–	–	–
Presence in milk	+	+	–	–	–
Selective secretion by seromucous glands	–	+	–	–	–
Heat stability (56°C)	+	+	+	+	–

* IgA may occur in 7S, 9S and 11S forms.

Immunoglobulin G (IgG)

This is the **main serum immunoglobulin**, constituting about 80 per cent of the total immunoglobulins. It has a molecular weight of 150,000 (7S). IgG may occasionally exist in a polymerised form. It is distributed equally between the intravascular and extravascular compartments. It contains less carbohydrate than other immunoglobulins. **IgG has a half-life of approximately 23 days**. When its level is raised, as in chronic malaria, kala azar or myeloma, the IgG synthesised against that particular antigen is catabolised rapidly and may result in the deficiency of the particular antibody. Conversely, in hypogammaglobulinemia, the IgG given for treatment is catabolised slowly. **The normal serum concentration of IgG is about 8–16 mg per mL.**

- IgG is the **only maternal immunoglobulin that is normally transported across the placenta** and provides natural passive immunity in the newborn. It is not synthesised by the fetus in any significant amount.
- IgG **binds to microorganisms and enhances their phagocytosis (by a mechanism known as opsonisation)**. Extracellular killing

of target cells coated with IgG antibody is mediated through the recognition of the surface *Fc* fragment by NK and K cells bearing the appropriate receptors.
- Interaction of IgG complexes with platelet *Fc* receptors probably leads to aggregation and vasoactive amine release.

IgG participates in most immunological reactions such as complement fixation, precipitation and neutralisation of toxins and viruses. It may be considered a general-purpose antibody, protective against infectious agents active in blood and tissues. **Passively administered IgG suppresses homologous antibody synthesis by a feedback process. This property is utilised in the isoimmunisation of women by the administration of anti-Rh(D) IgG during delivery**. With most antigens, IgG is a late antibody and makes its appearance after the initial immune response, which is IgM in nature.

Subclasses of IgG

It has four subclasses: IgG1, IgG2, IgG3 and IgG4, due to the presence of γ1, γ2, γ3 or γ4 H chains. Subtle differences are seen in the sequence of their constant regions. The subclasses differ from one another in the size of the hinge region and the number and position of the interchain disulphide bonds between the heavy chains. All four IgG subclasses are distributed in human serum in the approximate proportions of 65 per cent, 23 per cent, 8 per cent and 4 per cent, respectively.

Immunoglobulin A (IgA)

IgA is the second most abundant class of immunoglobulins, constituting about 10–13 per cent of serum immunoglobulins. The **normal serum level is 0.6–4.2 mg per mL**. It has a **half-life of 6–8 days.** It is the major immunoglobulin in the colostrum, saliva and tears. IgA occurs in two forms:

1. **Serum IgA** is principally a monomeric 7S molecule (MW about 160,000).
2. **Secretory IgA** (**dimeric SIgA**) found on mucosal surfaces and in secretions is a **dimer** formed by two monomer units joined together at their carboxy terminals by a glycopeptide termed the **J chain** (J for joining). This is synthesised by plasma cells situated near the mucosal

or glandular epithelium. The J chain is also produced in the same cells. J chains are also present in other polymeric immunoglobulins such as IgM. The schematic diagrams of all four classes are presented in Fig. 10.5.

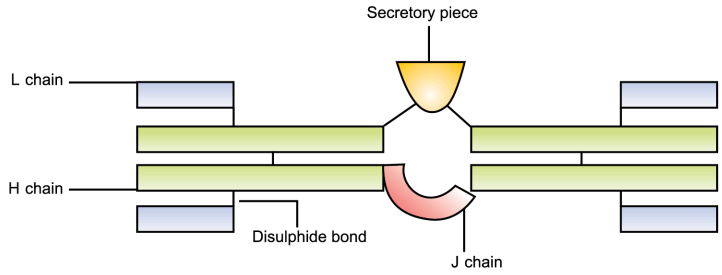


Fig. 10.5 Secretory IgA molecule

SIgA contains another glycine-rich polypeptide called the **secretory component** or **secretory piece**. This is not produced by lymphoid cells but by mucosal or glandular epithelial cells. Dimeric IgA binds to a receptor on the surface of the epithelial cells and is endocytosed and transported across the cells to the luminal surface. During this process, a part of the receptor remains attached to the IgA dimer. This part is known as the secretory component. **The secretory piece is believed to protect IgA from denaturation by bacterial proteases in sites such as the intestinal mucosa, which have rich and varied bacterial flora.** SIgA is a much larger molecule than serum IgA (11S; MW about 400,000).

SIgA is selectively concentrated in secretions and on mucous surfaces forming an **'antibody paste'** and is believed to play an important role in local immunity against respiratory and intestinal pathogens. Secretory IgA is relatively resistant to digestive enzymes and reducing agents. IgA antibodies may function by inhibiting the adherence of microorganisms to the surface of mucosal cells by covering the organisms and thereby preventing their entry into body tissues. This is done by cross-linking the multivalent antigen with polymeric IgA, and finally, the pathogen is eliminated.

- Secretory IgA provides an important defence mechanism against bacteria such as *Salmonella*, *Vibrio cholerae* and viruses such as polio, influenza, etc.
- Breast milk rich in IgA helps protect the newborn against infection during the first month of life. **Colostrum** contains large

amounts of S*IgA* that help protect the mucous membranes in the throat, lungs, and intestines of the infant.

- IgA does not fix complement but can activate the alternative complement pathway.
- It promotes phagocytosis and the intracellular killing of microorganisms.

Subclasses of IgA

Two IgA subclasses have been described: IgA1 and IgA2. IgA2 lacks interchain disulphide bonds between the heavy and light chains. Though IgA2 is a minor component of serum IgA, it is the dominant form in the secretions.

Immunoglobulin M (IgM)

IgM constitutes 5–8 per cent of serum immunoglobulins, with a **normal level of 0.5–2 mg per mL**. It has a **half-life of about five days**. It is a heavy molecule (19S; MW 900,000 to 1,000,000, hence called '**the millionaire molecule'**). **IgM molecules are polymers** of five four-peptide subunits, each bearing an extra C_H domain. As with IgA, polymerisation of the subunits depends on the presence of the J chain. Though the theoretical valency is 10, this is observed only with small haptens. With larger antigens, the effective valency falls to five, probably due to steric hindrance (Fig. 10.6). Monomeric IgM is the major antibody receptor on the surface of B lymphocytes for antigen recognition.

Most of **IgM (80 per cent) is intravascular in distribution**. Phylogenetically, IgM is the oldest immunoglobulin class**. It is also the earliest immunoglobulin to be synthesised by the fetus,** beginning by about 20 weeks of age.

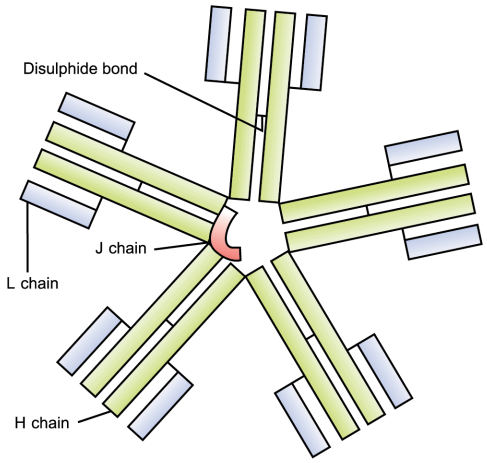


Fig. 10.6 IgM molecule

IgM is not transported across the placenta; the presence of IgM in the fetus or newborn indicates intrauterine infection and its detection is useful in the diagnosis of congenital infections such as syphilis, rubella, HIV infection and toxoplasmosis.

- IgM antibodies are relatively short-lived, disappearing earlier than IgG. Hence, their demonstration in serum indicates recent infection.
- Treatment of serum with 0.12 M 2-mercaptoethanol selectively destroys IgM without affecting IgG antibodies. This is a simple method for the differential estimation of IgG and IgM antibodies.
- The isohemagglutinins (anti-A, anti-B) and many other natural antibodies to microorganisms are usually IgM, as are antibodies to the typhoid 'O' antigen (endotoxin) and reagin antibodies in syphilis.

The unique structural features of IgM appear particularly suited to the biological role of providing protection against microorganisms and other large antigens that have repeating antigenic determinants on their surface**. A single molecule of IgM can bring about immune hemolysis,** whereas 1,000 IgG molecules are required for the same effect. IgM is also **500–1,000 times more effective than IgG in opsonisation**, 100 times **more effective in bactericidal action** and about 20 times more effective in **bacterial agglutination**. In the neutralisation of toxins and viruses, however, it is less active than IgG. Being largely confined to the intravascular space, IgM is believed to be responsible for protection against

blood invasion by microorganisms**. IgM deficiency is often associated with septicemias.**

Role of IgM in infectious diseases

As mentioned earlier, IgM is the first antibody produced as a primary response to an infection. It is seen in the early phase and appears to be involved in microbial infections. Because of its pentameric structure, it is able to bind to many viral particles and red blood cells. For this reason, smaller amounts of IgM are required to neutralise, opsonise and activate complement than IgG.

IgM detection in the serum denotes an early infection and often, may be diagnostic in certain infectious diseases, importantly the viral infections for which specific detection methods are absent.

Immunoglobulin D (IgD)

IgD resembles IgG structurally. It is present in a concentration of about **3 mg per 100 mL** of serum and is mostly intravascular. It has a **half-life of about three days.** IgD and IgM occur on the surface of unstimulated B lymphocytes and serve as recognition receptors for antigens. The combination of cell membrane-bound IgD or IgM with the corresponding antigen leads to specific stimulation of the B cell—either activation and cloning to produce antibody or suppression.

Immunoglobulin E (IgE)

It is an 8S molecule (MW about 190,000) with a **half-life of about two days**. It resembles IgG structurally. It exhibits unique properties such as heat lability (inactivated at 56°C in one hour) and **affinity for the surface of tissue cells** (**particularly mast cells**) of the same species (homocytotropism). IgE mediates the Prausnitz–Kustner (PK) reaction. It is susceptible to mercaptoethanol. It does not pass the placental barrier or fix complement. It is mostly extravascular in distribution. **Normal serum contains only traces** of IgE (a few nanograms per mL). Some of the characteristic features of IgE are:

- **Greatly elevated levels are seen in atopic (type 1 allergic) conditions** such as asthma, hay fever and eczema.

- Children living in unhygienic conditions with a high load of intestinal parasites have high serum levels of IgE.
- IgE is chiefly produced in the linings of the respiratory and intestinal tracts.

IgE deficiency has been associated with individuals with impaired immunity who present with undue susceptibility to infection. IgE is responsible for the anaphylactic type of hypersensitivity. The physiological role of IgE appears to be protection against pathogens by mast cell degranulation and release of inflammatory mediators. It is also believed to have a special role in defence against helminthic infections.

Overall, IgG protects the body fluids, IgA the body surfaces and IgM the bloodstream, while IgE mediates reaginic hypersensitivity. IgD is a recognition molecule on the surface of B lymphocytes.

Protective function of antibodies

Antibodies are responsible for humoral immunity. The close structural complementary resemblance to the antigen that provokes their synthesis binds the two on a non-covalent manner, which involves electrostatic Van der Waals and other weak forces. They protect against infection by the following mechanisms:

1. Phagocytosis By opsonising (coating) the organism. This is more pronounced against capsulated bacteria (e.g., pneumococci).
2. Virus neutralisation Antibodies prevent the binding of the virus to the receptors on the cells, preventing entry into the susceptible cell.
3. Complement-mediated lysis of cells Antibodies activate the complement pathway, which leads to complement-mediated cell lysis.
4. Antibody-mediated cell cytotoxicity They attach to the viral proteins on the cell surfaces of virally infected cells, which leads to interaction with the killer cells, resulting in lysis.
5. Neutralisation of toxins Exotoxins secreted by bacteria (e.g., tetanus toxin) are rendered inactive, thereby neutralising their toxic effects.

Antibodies also take part in active immunisation (vaccines induce their production) and passive immunisation (pre-formed antibodies injected into the host protect against toxins).

ABNORMAL IMMUNOGLOBULINS

Apart from antibodies, other structurally similar immunoglobulins are seen in serum in many pathological processes:

Multiple myeloma The earliest description of an abnormal

immunoglobulin was the discovery by Bence Jones (1847) of the protein that was named after him. The **Bence Jones protein** is typically found in multiple myeloma. It can be identified in urine by its characteristic property of coagulating when heated to 50°C and redissolving at 70°C. Bence Jones proteins are the light chains of immunoglobulins and therefore, may occur as the **kappa** or **lambda** forms. In any one patient, the chain is either kappa or lambda, and never both. **Myeloma is a plasma cell dyscrasia in which there is unchecked proliferation of one clone of plasma cells, resulting in the excessive production of the particular immunoglobulin synthesised by the clone**. The resulting disorder is, therefore, called **monoclonal Waldenstrom's macroglobulinemia.** Multiple myeloma may affect plasma cells synthesising IgG, IgA, IgD or IgE. Similar involvement of IgM-producing cells is known as Waldenstrom's macroglobulinemia. In this condition, there is excessive production of the respective myeloma proteins (**M proteins**) and of their light chains (**Bence Jones proteins).**

Heavy chain disease This is a form of paraproteinemia that causes lymphoid neoplasia and is characterised by the overproduction of the *Fc* parts of the immunoglobulin heavy chains. Three types of heavy chain disease (HCD) are recognised based on the class of immunoglobulin heavy chain produced (alpha, gamma or mu) by the malignant cell.

Cryoglobulinemia This is a condition in which a gel or precipitate is formed on cooling the serum, which redissolves on warming. It may not always be associated with disease but is often found in myelomas, macroglobulinemias and autoimmune conditions such as systemic lupus erythematosus. Most cryoglobulins consist of IgG, IgM or their mixed precipitates.

The monoclonal natures of Bence Jones and other M proteins have been valuable models for the understanding of immunoglobulin structure and function.

IMMUNOGLOBULIN SPECIFICITIES

Immunoglobulin specificity is important in immunology. The antigenic determinants, or **paratope**, on immunoglobulin molecules fall into three main categories and are located in characteristic portions of the molecule (Fig. 10.7).

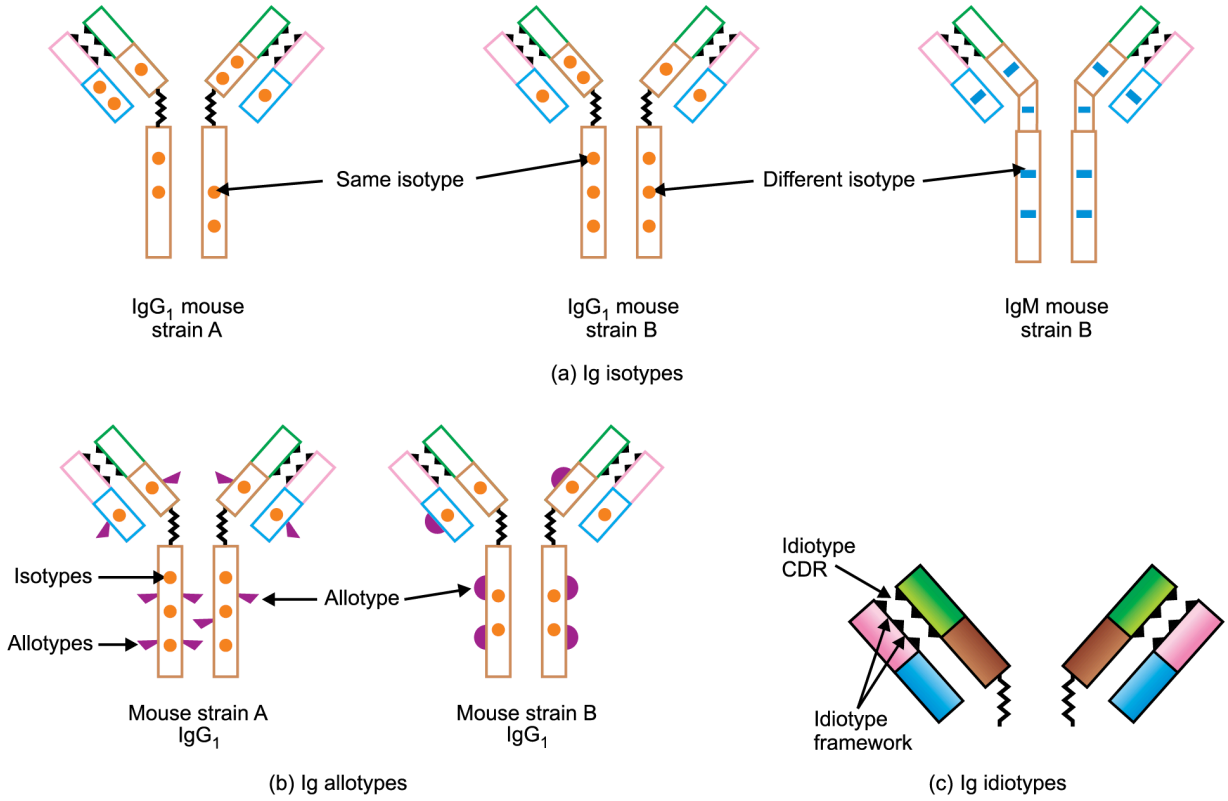


Fig. 10.7 Isotypes, allotypes and idiotypes of antibodies

Isotype

Isotypic determinants refer to **the genetic variations or differences in the constant region of the heavy chain** of the Ig classes and subclasses within a species. Each isotype is encoded by a separate constant-region gene, and all members of a species carry the same constant-region genes. Within a species, each normal individual expresses all isotypes in the serum. Different species inherit different constant-region genes and, therefore, express different isotypes. When an antibody from one species is injected into another, the isotypic determinants are recognised as foreign, inducing an antibody response to the isotypic determinants.

Allotype

Allotypic determinants refer to **multiple alleles that exist for some genes** and lead to subtle amino acid differences in some, but not all members of a species. The sum of the individual allotypic determinants displayed by an antibody determines its allotype. In humans, allotypes have been characterised for all four IgG subclasses, for one IgA subclass, and for the

kappa light chain**. Each of these allotypic determinants represents differences in one to four amino acids that are encoded by different alleles**. Antibodies to allotypic determinants can be produced by injecting antibodies from one member of a species into another member of the same species. However, such an antibody carries different allotypic determinants. **Antibody to allotypic determinants is sometimes produced by a mother during pregnancy in response to paternal allotypic determinants on the fetal immunoglobulins**. Antibodies to allotypic determinants can also arise from a blood transfusion. To date, in the human system, no allotypic markers have been found for γ light chains or ∝, δ and ε heavy chains.

Idiotype

Idiotypic determinants arise from the sequence of the heavy and light chain variable regions. **Each individual antigenic determinant of the variable region (paratope) is referred to as an idiotope**. The sum total of **idiotopes** on an Ig molecule constitutes its idiotype. An idiotope may be the actual antigen-binding site or variable region sequences outside of the antigen-binding site. By immunisation with *Fab* fragments, anti-idiotypic antibodies can be produced. These resemble the epitopes of the original antigen. Used as a vaccine, the anti-idiotypes provide protection against the original antigen (pathogen or tumour) in experimental animals. **Sequential anti-idiotypic antibody formation is the basis of Jerne's network hypothesis of immune regulation.**

ANTIBODY DIVERSITY

One of the important structure–function relationships to antigen recognition is antibody diversity.

An individual is capable of making an enormous number of structurally distinct antibodies, each with distinct specificity. The presence of a large number of antibodies that bind different antigens/pathogens is called **antibody diversity**, and the total collection of antibodies with different specificities is called the **antibody repertoire**. This diversity is mainly of genetic origin, where the recombination of different gene segments plays an important role. The genetic mechanisms that can generate such a **large antibody repertoire occur exclusively in lymphocytes**.

Clinical significance of antibody diversity

The knowledge of immunoglobulin genes has made it possible to develop engineered antibodies for therapeutic purposes to treat various lymphomas and autoimmune diseases. In human immunodeficiency virus (HIV), the genetic variation and mutability of the virus has created a plethora of constantly changing antigens that generate diverse immune responses. This poses a major challenge in developing an HIV vaccine.

CLASS SWITCHING

On antigen stimulation, the plasma cells produce a particular antibody (e.g., IgM). Later, gene arrangement allows for the same antigen specificity but a different immunoglobulin class (e.g., IgG or IgA). The H chain VDJ unit can join any constant gene segment (for heavy chain) and express the particular antibody class. This process is called **class switching**. The exact mechanism is unclear but evidence suggests that the V_H- gene can sequentially associate with the C_H gene segment (except C_δ). These are called **switch sites (SS)** and are composed of multiple copies of short repeated sequences (GAGCT and TGGGG). Class-specific recombinases bind to switch sites and facilitate recombination. Therefore, the expression of a particular antibody class depends on the specificity of the recombinase proteins expressed.

Class switching happens after antigenic stimulation and depends on the interplay of three factors:

1. Switch regions/sites
2. Switch recombinase
3. Cytokine signals (IL-4)

Clinical significance of class switching

Immunoglobulin class switch recombination deficiencies (Ig CSR deficiencies) or hyper IgM syndromes (HIGM) are a group of primary immunodeficiency diseases characterised by defective CD40 signalling of B cells. Affected patients are characterised by low serum levels of IgG and IgA, and normal or elevated levels of IgM, which lead to increased susceptibility to infections such as bacterial infections of the skin and respiratory tract, mucosal ulcers and diarrhea.

GENETIC REGULATION OF IMMUNOGLOBULINS

B cells and their lineage express the immunoglobulin genes. Two major classes of regulatory sequences in the immunoglobulin DNA regulate the transcription of genes. They are:

i) Promoters, which are a group of short nucleotide sequences that initiate transcription

ii) Enhancers, which activate transcription from the promoter sequence

Immunoglobulins are unique in their property to respond to an array of foreign antigens. Variations in heavy and light chain proteins depend on the immunoglobulin genes. Multiple genes encode for a single immunoglobulin heavy or light chain. During maturation in the B cell, the genes are reorganised in an ordered sequence (of Ig gene) to contribute to the diversity of the final product. The control is by a complex process which includes organisation of the immunoglobulin genes, followed by Ig gene rearrangement and a dynamic process of generating 10^8 different antigenic specificities in an antibody.

Knowledge of antibody gene sequence and the structure of antibodies along with molecular technology has made it possible to design and construct genes that code for formation of immunoglobulins, the **man-made antibody,** using hybridoma technology (discussed in the following paragraphs).

MONOCLONAL ANTIBODIES

Principle

A single antibody-forming cell or clone produces antibodies directed specifically against a single antigen or antigenic determinant only. When a clone of lymphocytes or plasma cells undergoes selective proliferation, as in multiple myeloma, antibodies with single antigenic specificity accumulate. Such antibodies produced by a single clone and directed against a single antigenic determinant are called **monoclonal antibodies**.

An ingenious method for the large-scale production of monoclonal antibodies against any desired antigen was developed by Köhler and Milstein in 1975. In recognition of the great importance of this hybridoma technology, the Nobel Prize for Medicine was awarded to them in 1984.

Hybridomas are somatic cell hybrids produced by fusing antibody-forming spleen cells with myeloma cells. The resultant hybrid retains the

antibody-producing capacity of the spleen cell and the ability of the myeloma cells to multiply indefinitely (Fig. 10.8).

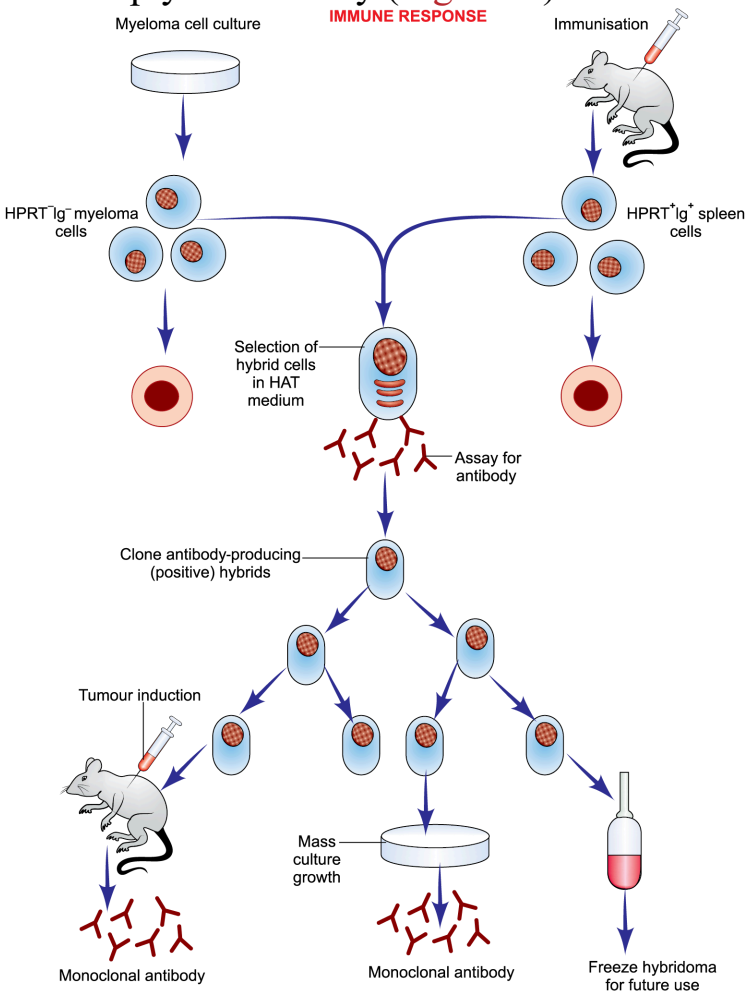


Fig. 10.8 Monoclonal antibody production by hybridoma technique

Technique

Lymphocytes from the spleen of mice immunised with the desired antigen are fused with mouse myeloma cells that are deficient in the enzyme hypoxanthine phosphoribosyl transferase (HPRT). These cells are capable of replicating indefinitely. The fused cells are placed in a medium containing **hypoxanthine, aminopterin and thymidine (HAT)**. This does not permit the growth of enzyme-deficient myeloma cells. Only hybrid cells possessing properties of both the splenic lymphocytes and myeloma cells grow in the culture. These hybrid cells called **hybridomas** are cloned and examined for the production of antibodies. Clones producing antibodies against the desired antigen are selected for continuous cultivation. These

hybridomas can be maintained indefinitely in culture and will continue to form monoclonal antibodies. They can also be injected intraperitoneally in mice; monoclonal antibodies may be obtained by harvesting the ascitic fluid of mice. Hybridomas may then be frozen for prolonged storage.

The discovery of hybridoma technology for the production of unlimited quantities of identical monoclonal antibodies of the same Ig class, possessing uniform specificity, affinity and other properties, revolutionised clinical immunology.

Applications of monoclonal antibodies

Monoclonal antibodies have important clinical uses:
- Therapeutic—for treatment of lymphomas, rheumatoid arthritis, etc.
- Diagnostic imaging—radiolabelled monoclonal antibodies are used to detect tumour antigens
- Diagnostic reagents—diagnosing infections (immunoassays) and measuring blood levels of various drugs
- Research—in vitro production of immunoglobulins

HUMANISED (CHIMERIC) ANTIBODIES

Mouse monoclonals have been humanised by genetic manipulation to make chimeric antibodies consisting of **murine variable regions and human constant regions**. Grafting of murine monoclonal CDR loops on a human Ig framework provides a virtually human molecule. (The antigen-binding surface of an antibody is composed of six hypervariable loops of amino acids. These are called **complementarity-determining regions** or CDRs.)

ANTIBODY ENGINEERING

Whole libraries of antibodies have been built by fusing genes coding for antibodies with bacteriophage genomes. Large quantities of the desired human antibody can be prepared by infecting bacteria with the appropriate bacteriophage. Such antibody engineering holds great promise for immunotherapy.

Key points

- An antibody (immunoglobulin) is a glycoprotein molecule formed by the immune system in response to an antigenic stimulus; it is found in blood, bodily secretions or on mucous

surfaces, and binds to the specific antigen responsible for its production, thereby inactivating it.

- There are five classes of immunoglobulins: IgG, IgM, IgA, IgD and IgE; antibodies of all five classes can combine specifically with antigens.
- IgG constitutes 80–85 per cent of the total amount of antibodies; it can cross the placenta, fix complement and specifically attach to phagocytes. It provides protection within the body, causes lysis or removal of foreign antigens and neutralises viruses and toxins.
- IgM constitutes 9–10 per cent of the total amount of antibodies. It fixes complement but is unable to cross the placenta or attach to phagocytes. It provides protection within the body and is especially effective in agglutinating antigens and in inactivating complement.
- IgA constitutes about 10 per cent of total antibody. It is secreted into external secretions (saliva, milk, mucus) and guards mucosal surfaces.
- IgD constitutes 1–3 per cent of the total amount of antibodies. It serves as an antigen receptor on B lymphocytes and promotes the development and maturation of the antibody response.
- IgE constitutes just 0.05 per cent of total antibody. It specifically attaches to mast cells and basophils, and is involved in type I hypersensitivity (allergic and atopic) reactions.
- Abnormal immunoglobulins include Bence Jones proteins in multiple myeloma, overproduction of the *Fc* parts of the immunoglobulin heavy chains in heavy chain disease and cryoglobulins consisting of IgG, IgM or their mixed precipitates in cryoglobulinemia.
- Isotypic (genetic variations or differences in the constant region of the heavy chain), allotypic (multiple alleles for some genes) and idiotypic (sequence of the heavy and light chain variable regions) antigenic determinants are located on immunoglobulin molecules and play an important role in antibody specificity.
- The presence of a large number of antibodies that bind different antigens/pathogens is called antibody diversity.
- On antigen stimulation, the H-chain VDJ unit can join any constant heavy gene segment and express that particular antibody class. This process is called class switching.

Questions

Essays

1. Define an antibody. Draw a labelled diagram of an immunoglobulin with its different domains.
2. Describe the various classes of immunoglobulins and list their properties and functions.

Short Answers

1. Effect of enzymatic (papain and pepsin) digestion on immunoglobulin molecules
2. Structure of the IgM molecule
3. Structure of the IgG molecule
4. Structure of IgA molecule with diagram
5. Abnormal immunoglobulins
6. Isotypes

7. Monoclonal antibodies and their uses

Part II: Immunology

Chapter 11

Complement System

Chapter navigation

- Properties and functions of complement
- Complement pathways
  - Classical pathway
  - Alternate pathway
  - Lectin pathway
- Regulation of complement

INTRODUCTION

The term complement (C) refers to a system of soluble and cell-bound proteins that occur in normal serum, and are activated by antigen–antibody interaction. They subsequently mediate a number of biologically significant consequences.

The term **complement** was coined by Ehrlich based on the fact that this factor complemented the action of the antibody.

Bordet and Gengou (1901), using the hemolytic indicator system, described the **complement fixation test** as a sensitive serological reaction.

The complement system belongs to a group of biological effector mechanisms (called **triggered enzyme cascades**), which also include coagulation and the fibrinolytic and kinin systems. Each enzyme in the cascade can activate many molecules of the succeeding component, resulting in the amplification of the response at each step. Every step has its own control mechanisms so that the cascade can be regulated with precision.

PROPERTIES OF COMPLEMENT

- Complement is present in the sera of all mammals and also in that of most other animals, including birds, amphibians and fish. It is a non-specific serological reagent in that the complement from one species can react with antibodies from other species. **Complement constitutes about 5 per cent of normal serum protein and does not increase as a result of immunisation.**
- Though some of its components are heat-stable, complement as a whole is heat-labile; its cytolytic activity undergoes spontaneous denaturation slowly
  at room temperature and **is destroyed in 30 minutes at 56°C**.
- A serum deprived of its complement activity by heating at 56°C for 30 minutes is said to be '**inactivated'.**
- Complement (C) ordinarily does not bind to free antigens or antibodies but only to antigen–antibody complexes. Various terms such as **fixation, binding** and **consumption** are used to refer to the combination of C with bound immunoglobulin. This leads to the activation of the **classical C pathway**.
- **All classes of Ig do not fix C**. Only IgM, IgG_3, IgG_1 and IgG_2 (in that order) fix C; IgG_4, IgA, IgD or IgE do not. The site of C binding is located on the *Fc* piece of the Ig molecule (C_H2 domain on IgG and C_H4 on IgM). The binding site (*Fc*) is expressed only when Ig is combined with its antigen. **The fixation of C is not influenced by the nature of antigens but only by the class of immunoglobulins.**

COMPONENTS OF COMPLEMENT

The complement system **consists of at least 30 chemically and immunologically distinct serum proteins which make up the complement components;** these include:

1. Properdin system
2. Control proteins

The biological activities of this system affect both innate and acquired immunity. Interactions of cellular receptors with C proteins control B cell activation and play a crucial role in the highly developed acquired immune

system.

Fractions
Complement is a complex of **nine** different fractions, **C1 to C9**. The fraction C1 occurs in serum as a calcium ion-dependent complex, which on chelation with EDTA, yields three protein subunits called C1q, r and s. Thus, C is made up of a total of 11 different proteins. C fractions are named C1 to C9 in the sequence of the cascading reaction, except that C4 comes after C1 and before C2.

The model traditionally used to explain C activity in immune cytolysis is the lysis of erythrocytes sensitised by its antibody. **The erythrocyte–antibody complex is called EA. When C components are attached to EA, the product is called EAC**, followed by the components that have reacted (for example, EAC 14235 or EAC 1–5). When a C component acquires enzymatic or other demonstrable biological activity, it is indicated by a bar over the component number, for example, enzymatically activated C1 is shown
as C1–. Fragments cleaved from C components during the cascade are indicated by small letters (e.g., C3a, C3b). Inactivated forms of C components are indicated by the prefix 'i' (e.g., iC3b).

Functions of the complement

1. Cytolysis—lysis of cells, bacteria and virus by the membrane attack complex
2. Chemotaxis—the movement of phagocytic cells towards bacterial cells with the help of C5a (potent chemotactic agent)
3. Opsonisation—C3b receptors on phagocytes render the process of phagocytosis more efficient
4. Binding of complement components to cells trigger
   – Inflammatory reactions
   – Specific cell functions
   – Secretion of immunoregulatory molecules
5. Immune clearance removes immune complexes and deposits them in the liver and spleen

COMPLEMENT ACTIVATION

Complement is normally present in the body in an inactive form but when its activity is induced by antigen–antibody combination or other stimuli, C components react in a specific sequence as a cascade. This phenomenon is called complement activation. Basically, the **C cascade** is a series of reactions in which the preceding components act as enzymes on the succeeding components, cleaving them into dissimilar fragments. The larger fragments usually join the cascade. The smaller fragments that are released

often have biological effects which contribute to defence mechanisms by various basic **effector mechanism** functions mentioned above.

The **resultant effects** are the following (Fig. 11.1):

- Amplication of the inflammatory process
- Increase in vascular dilation and permeability (by anaphylatoxin, c3a and c5a)
- Inducing of smooth muscle contraction
- The release of histamine from mast cells

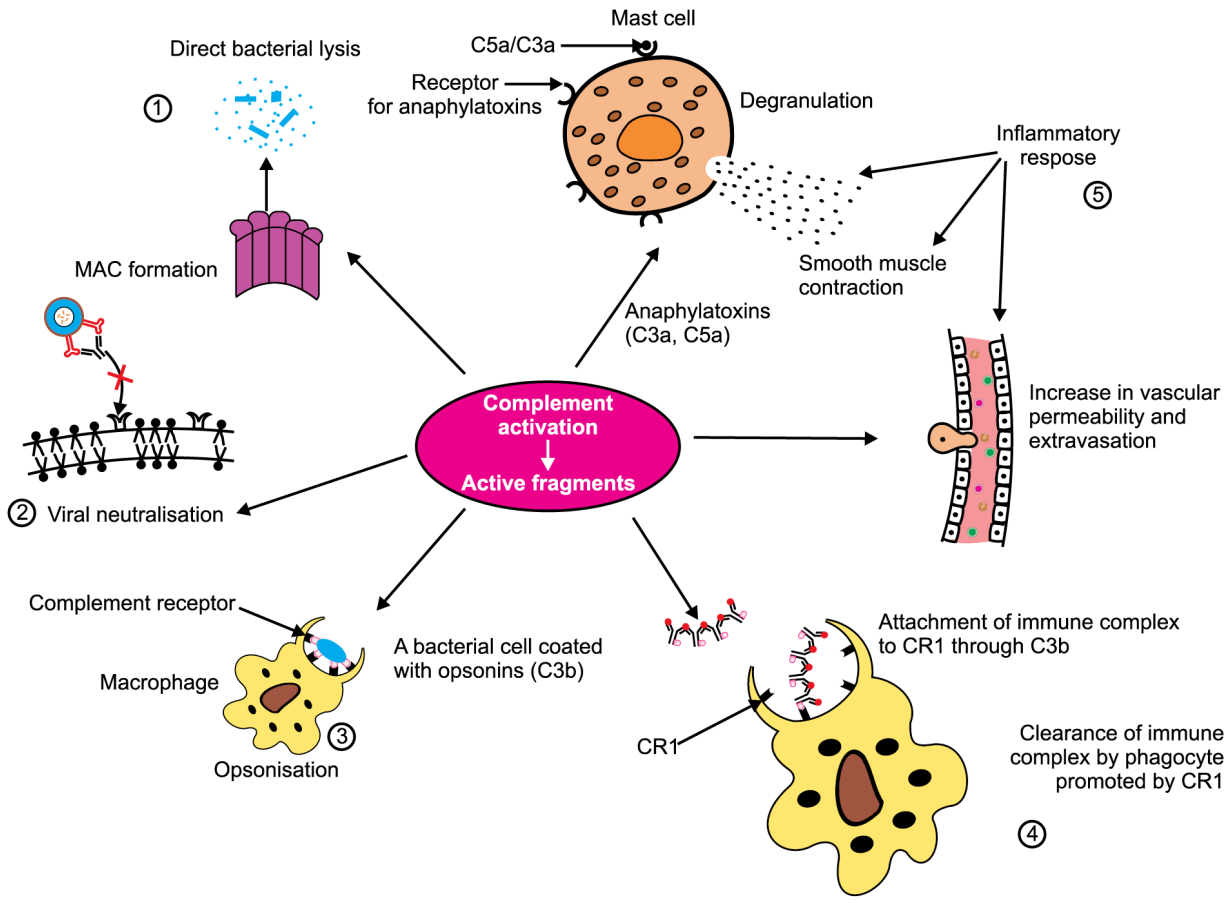


Fig. 11.1 Biological effects of complement activation

COMPLEMENT PATHWAYS

The C cascade can be triggered by **three parallel but independent mechanisms or pathways** which differ only in the initial steps. Once C3 activation occurs, the subsequent steps are common to all pathways. The three pathways are the following:

1. Classical C pathway
2. Alternative or properdin pathway
3. Lectin pathway

The classical pathway is so called because it was the first one identified. It is a more recently evolved mechanism of specific active immunity, while the alternative pathway and lectin pathway represent a more primitive system of non-specific innate immunity.

1. Classical Complement Pathway

The chain of events in which complement components react in a specific sequence following activation of C1qrs, as mentioned below, and typically culminate in immune cytolysis is known as the classical pathway. Classical pathway is generally activated by the (a) antigen–antibody complex, (b) aggregated immunoglobulin, (c) DNA, (d) C-reactive protein, (e) trypsin-like enzymes and (f) some retroviruses.

The **steps of the classical pathway are as follows** (Fig. 11.2):

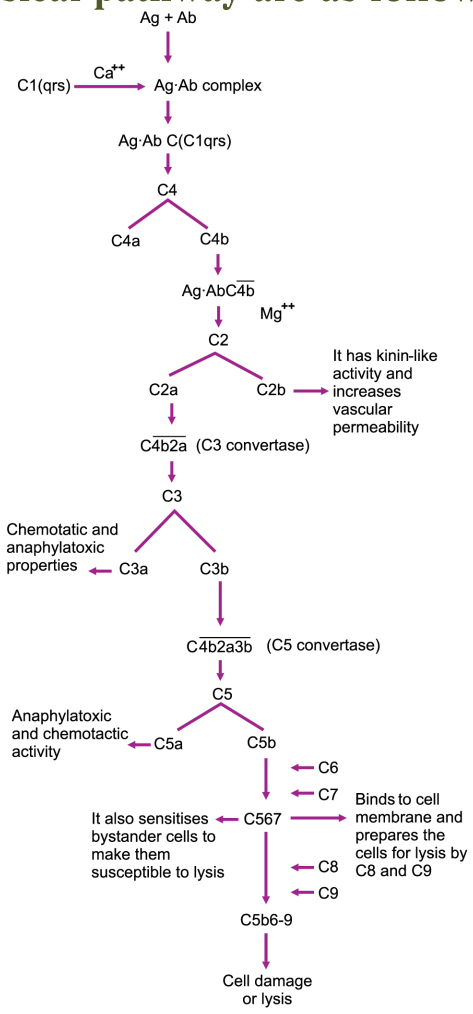


Fig. 11.2 The classical pathway of complement

i) The first step is the **binding of C1 to the antigen–antibody complex** (traditionally represented as EA). C1 is the first complement component to participate in the classical pathway. It is composed of C1q, C1r and C1s. The recognition unit of C1 is C1q, which reacts with the *Fc* piece of bound IgM or IgG. C1q has six combining sites. Effective activation occurs only when C1q is attached to immunoglobulins by at least two of its binding sites. One molecule of IgM or two molecules of IgG can, therefore, initiate the process. C1q binding in the presence of calcium ions leads to sequential activation of C1r and C1s leading to the **C1qrs** complex.

ii) C1–s– is an esterase (C1–s– esterase), one molecule of which can cleave several molecules of C4—an instance of amplification. C4 is split into C4a, which is an anaphylatoxin, and C4b, which binds to cell membranes along with C1.

iii) C4–b–, in the presence of magnesium ions cleaves C2 into C2a, which remains linked to cell-bound C4–b–, and C2b that is released into the fluid phase. C4–b–2–a– has enzymatic activity and is referred to as the classical pathway **C3 convertase**.

iv) C3 convertase splits C3 into two fragments: C3a, which is an anaphylatoxin and C3b, which remains cell-bound along with C4–b–2–a– to form a trimolecular complex C4–b–2–a–3–b–. This complex has enzymatic activity and is called C5 convertase.

v) The **membrane attack phase** of complement activity begins at this stage, with C5 convertase cleaving C5 into C5a, an anaphylatoxin which is released into the medium, and C5b which continues with the cascade. C6 and C7 then join together. A heat-stable trimolecular complex C5–6–7– is formed, part of which binds to the cell membrane and prepares it for lysis by C8 and C9 which join the reaction subsequently. Most of C5–6–7– complexes escape and serve to amplify the reaction by adsorbing onto unsensitised '**bystander cells**', and rendering them susceptible to lysis by C8 and C9. The unbound C5–6–7– has chemotactic activity, though the effect is transient due to its rapid inactivation. The mechanism of complement-mediated cytolysis is the production of 'holes',

approximately 100 Å in diameter, on the cell membrane. This disrupts the osmotic integrity of the membrane, leading to the release of the cell contents.

2. Alternative Complement Pathway

This pathway is antibody-independent. **The activation of C3 without the prior participation of C4–b–2–a– is known as the alternative pathway.**

Many substances can activate the pathway. These activators include:

i) Bacterial endotoxins and teichoic acid of gram-positive bacteria
ii) IgA and D
iii) Cobra venom factor and the nephritic factor (a protein present in the serum of glomerulonephritis patients)
iv) Zymosan (polysaccharide from yeast cell wall)
v) Some virus and virus-infected cells
vi) Parasites, e.g., trypanosomes

The **steps of the alternative pathway are as follows** (Fig. 11.3):

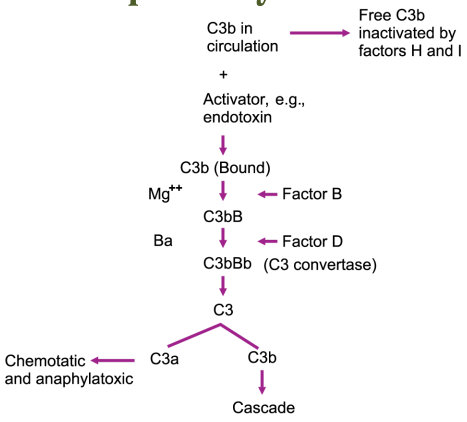


Fig. 11.3 Alternative pathway of complement

i) The first step in the alternative pathway is the **binding of C3b** to an activator. C3b is continuously generated in small quantities in circulation but in the free state, it is rapidly inactivated by serum protein factors H and I.
ii) Bound C3b, which is protected from such inactivation, interacts with a serum protein called Factor B (also known as '**C3 proactivator**') to form a magnesium-dependent complex 'C3b,B'.
iii) This complex is cleaved by another serum protein, Factor D (also called '**C3 proactivator convertase**'), into two fragments, Ba and Bb.

Fragment Ba is released into the medium. Fragment Bb remains bound to C3b, forming the esterase C3–b–B–b– complex, which is the alternative pathway C3 convertase. This enzyme is extremely labile. The function of properdin (also called Factor P) is to stabilise the C3 convertase, which hydrolyses C3, leading to further steps in the cascade, as in the classical pathway (Fig. 11.2).

3. Lectin Complement Pathway

This involves the host protein binding to the microbial surface. The proteins recognise and bind to specific carbohydrate targets. Since the lectin that activates complement binds to mannose residues, it is also called **mannose-binding lectin** or **MB lectin (MBL),** an acute-phase protein produced in inflammatory responses. Its function is similar to that of C1q. The pathway is the **mannan-binding lectin pathway**. It does not depend on antibody for its activation.

The lectin pathway is activated by the binding of MBL to mannose residues present on the surfaces of microorganisms like certain *Salmonella*, *Neisseria*, *Listeria Cryptococcus* and *Candida*. The steps in the lectin complement pathway are as follows:

i) MBL binds to the foreign surface of a pathogen.
ii) Proteases (MASP1 and MASP2) bind to it and form the C1-like active complex.
iii) Cleavage and activation of C4 and C2 occur to produce C5 convertase without the need for specific antibody binding.

Lectin pathway represents an innate defence mechanism comparable to the alternative pathway while utilising classical pathway components except the C1 proteins.

The three complement pathways converge at the membrane attack complex (MAC), which contains C5b, C6, C7, C8 and C9 proteins after sequential interaction to form this macromolecular structure.

MAC forms a large channel through the membrane of a pathogen or target cell, enabling ions and small molecules to diffuse freely across the membrane. As a result, the cell cannot maintain its osmotic stability and is killed by an influx of water and loss of electrolytes. An overview of all three pathways is represented in

Fig. 11.4.

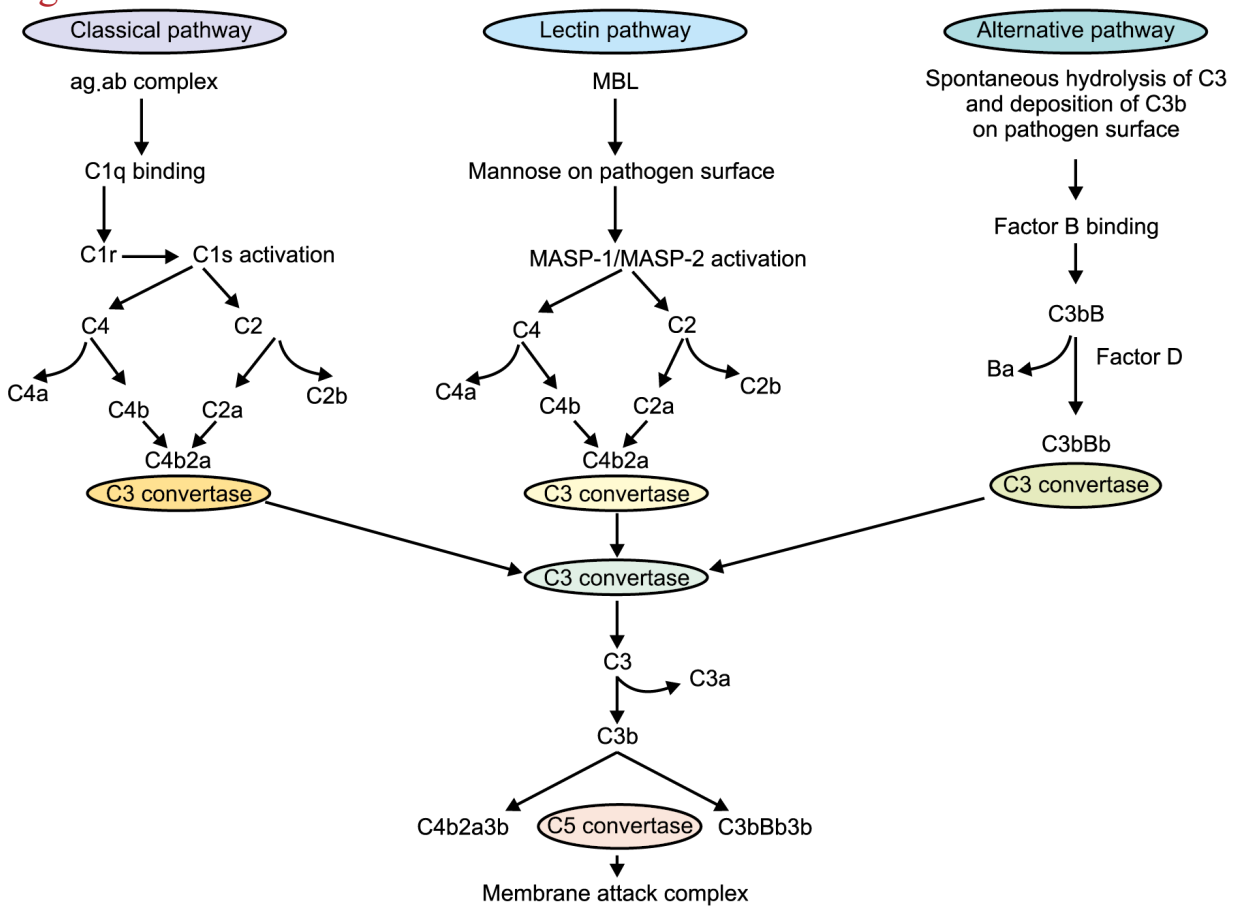


Fig. 11.4 Overview of pathways of complement activation

REGULATION OF COMPLEMENT ACTIVATION

Unchecked complement activity can cause not only exhaustion of the complement system but also serious damage to tissues. Several in-built control mechanisms regulate the complement cascade at different steps. These are mainly of two kinds:

1. **Inhibitors**, which bind to complement components and halt their further function
2. **Inactivators**, which are enzymes that destroy complement proteins

Inhibitors

- C1q inhibitors bind to and inactivate serine protease activity of C1r and C1s.

- Factor 1 cleaves C3b and C4b, reducing the amount of C5 convertase.
- Factor P (properdin) stabilises C3 convertase and protects C3b.
- S protein binds to C6–7– and modulates the cytolytic action of the membrane attack complex.

Inactivators

- **Factor H** (beta globulin) acts in concert with Factor I, modulating C3 activation.
- **Anaphylatoxin inactivator** (alpha globulin) enzymatically degrades C3a, C4a and C5a, which are anaphylatoxins released during the C cascade.
- The **C4 binding protein** controls the activity of cell-bound C4b.

BIOLOGICAL EFFECTS OF COMPLEMENT ACTION

Complement mediates immunological membrane damage (cytolysis, bacteriolysis), amplifies the inflammatory response and participates in the pathogenesis of certain hypersensitivity reactions. It exhibits antiviral activity and promotes phagocytosis and immune adherence. Listed below are some of the biological effects of complement activation on the host:

1. ***Phagocytosis*** An important function of C is to facilitate the uptake and destruction of pathogens by phagocytic cells. This opsonic effect is based on the presence of phagocytic cells on the surface (macrophages, monocytes, neutrophils and others) of complement receptors or CRs. Many such receptors have been identified, such as CR 1, 2, 3, 4 and C1q, which stimulate phagocytosis and the removal of immune complexes. The CR2 receptor on B cells also acts as a receptor for the Epstein–Barr virus (EBV), the causative agent of infectious mononucleosis, and so has a role in the pathogenesis of this condition.
2. ***Inflammatory response*** C fragments released during the cascade reaction help in amplifying the inflammatory response. C2 kinins are

vasoactive amines and increase capillary permeability. C3a and C5a are anaphylatoxic (histamine-releasing) and chemotactic. C5–6–7– is chemotactic and also brings about reactive lysis.

3. ***Hypersensitivity reactions*** C participates in **cytotoxic (Type II)** and **immune complex (Type III)** hypersensitivity reactions. The destruction of erythrocytes, following incompatible transfusion, and **thrombocytopenia** in sedormid purpura are examples of Type II reactions. C contributes to the pathogenesis of **nephrotoxic nephritis**, though immunological kidney damage may also occur in its absence. C is required for the production of immune complex diseases such as **serum sickness** and **Arthus reaction**.
4. ***Autoimmune diseases*** Serum C components are decreased in many autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. C also plays a major role in the pathogenesis of **autoimmune hemolytic anemia.**
5. ***Endotoxic shock*** Endotoxin is an efficient activator of the alternative C pathway. In endotoxic shock, there is massive C3 fixation and platelet adherence. Large-scale platelet lysis and the release of large amounts of platelet factors lead to **disseminated intravascular coagulation** (DIC) and thrombocytopenia. Gram-negative septicemias and **dengue hemorrhagic syndrome** may have a similar pathogenesis. Depletion of C protects against the Shwartzman reaction.
6. ***Immune adherence*** C bound to antigen–antibody complexes adheres to erythrocytes or to non-primate platelets. This reaction, called immune adherence, contributes to defence against pathogenic microorganisms since such adherent particles are rapidly phagocytosed. C3 and C4 are necessary for immune adherence.

Conglutination

Bovine serum contains an unusual protein called conglutinin (K) which causes the clumping of particles or cells coated with C, a process known as conglutination. Conglutinin reacts exclusively with bound C3. Though conglutinin behaves as an antibody to C, it is not an immunoglobulin and requires Ca++ for its activity. Antibodies with conglutinin-like activity (immunoconglutinin, IK) can be produced by immunisation with complement-coated materials. They may also occur frequently in human beings and other mammals as autoantibodies to fixed C.

The complement system is generally effective in lysing gram-negative bacteria; however, some gram-negative bacteria and most gram-positive bacteria have mechanisms to evade complement-mediated damage, as shown in Table 11.1.

Table 11.1 Microbial evasion of complement-mediated damage

Microbial components	Mechanism of evasion	Examples
Gram-negative bacteria		
Cell wall lipopolysaccharides (LPS)	Side chains prevent insertion of MAC into bacterial membrane	Resistant strains of *E. coli* and *Salmonella*
Other membrane proteins	MAC interacts with membrane proteins and fails to insert into bacterial membrane	Resistant strains of *Neisseria gonorrhoeae*
Elastase	Anaphylatoxins C3a and C5a are inactivated by microbial elastase	*Pseudomonas aeruginosa*
Gram-positive bacteria		
Peptidoglycan layer of cell wall	Thick layer prevents MAC from inserting into the bacterial membrane	*Streptococcus*
Bacterial capsule	Provides physical barrier between C3b deposited on bacterial membrane and CR1 on phagocytic cells	*Streptococcus pneumoniae*
Other microbes		
Proteins that mimic complement regulatory proteins	Proteins present in various bacteria, viruses, fungi and protozoans inhibit the complement cascade	Vaccinia virus, herpes simplex, Epstein–Barr virus, *Toxoplasma cruzi, Candida albicans*

Complement components need to be estimated in (a) acquired and inherited complement deficiencies, (b) inherited and acquired C1 inhibitor deficiencies and (c) disorders with complement activation.

- ***Estimation of individual complement components*** The concentration of individual proteins is determined by various types of immunoprecipitation assays (nephelometry and turbidimetry).
- ***Detection of activation products*** Complement activation products are detected by identifying C3a or C3d.
- ***Estimation of complement function*** Hemolytic assays utilising sheep erythrocytes coated with rabbit antibodies (IgM with or without IgG) are used to estimate complement function.

Recently, solid-phase functional tests have been introduced to detect all three functional activities of complement by enzyme-linked immunosorbent assays (ELISA).

Complement activity of serum is measured by estimating the highest dilution of serum required to lyse sheep erythrocytes sensitised by anti-erythrocytic antibody. Estimation of individual complement components also uses **hemolytic activity in systems** containing an excess of all complement components except the one to be measured.

BIOSYNTHESIS OF COMPLEMENT

Complement components are synthesised at various sites in the body such as the **intestinal epithelium** (C1**), macrophages (**C2, C4), **spleen** (C5, C8) and **liver** (C3, C6, C9).

C is an **acute phase reactant** and a rise in C levels (particularly C4, C3, C5 and C6) is observed during the acute phase of inflammation.

DEFICIENCIES OF THE COMPLEMENT SYSTEM

Complete or partial deficiencies of all classical complement components and several of the C inhibitors have been described in humans and animals. Some are associated with severe diseases, while in others, clinical manifestations are sporadic. C deficiencies result in the host being unable to efficiently eliminate the microbial antigens or circulating immune complexes. Recurrent bacterial and fungal infections and collagen diseases also occur (Table 11.2).

Table 11.2 Clinical syndromes associated with genetic deficiencies of complement components

Group	Deficiency	Syndrome
I	C1 inhibitor: autocatalytic activation of C1 and the unrestrained breakdown of C4 and C2	Hereditary angioneurotic edema (episodic angioedema of subcutaneous tissues or of the mucosa of the respiratory or alimentary tracts)
II	Early components of classical pathway C1, C2, C4	SLE and other collagen vascular diseases
III	C3 and its regulatory protein C3b inactivator	Severe recurrent pyogenic infections
IV	C5 to C8	Bacteremia, mainly with gram-negative diplococci, oxoplasmosis
V	C9	No particular disease

Key points

- The complement system consists of at least 30 distinct proteins. The major components are C1 through C9, which are numbered in the order of their discovery, not in the order in which they react.
- Some of the proteins of the complement system are inactive enzymes which, once activated, act in sequence, one after another in a cascade reaction. Other components perform specific biologic functions.
- The components released after activation often possess various biological effector functions which contribute to defence mechanisms such as lysis of cells and bacteria, promoting virus neutralisation, opsonisation (which promotes phagocytosis of particulate antigens) and immune clearance (which removes immune complexes from the circulation).
- Complement can be activated in three ways: by the classical pathway, which requires a specific immune reaction for activation and by the alternative pathway and lectin pathway, which are antibody independent. These pathways are activated by the reaction of complement proteins with surface molecules of microorganisms.
- The complement destroys invading bacteria and foreign cells by disrupting their cytoplasmic membranes and is also involved in the inflammatory response since it contributes to vascular permeability, stimulates chemotaxis and enhances phagocytosis.
- Complement proteins and protein fragments with receptors on the cells of the immune system control both innate and acquired immune response.

- The direct and indirect complement fixation tests are examples of in vitro diagnostic antigen–antibody reactions.
- Complement deficiencies result in the host being unable to efficiently eliminate the microbial antigens or circulating immune complexes, causing recurrent bacterial and fungal infections.

QUESTIONS

Essays

1. Describe the basic properties of the complement system and the classical complement pathway.
2. Briefly explain the alternative pathway of the complement system.

Short Answers

1. Biological functions mediated by the complement system
2. Activators and inhibitors for the regulation of the complement system
3. Classical complement pathway
4. Alternative complement pathway (properdin pathway)
5. Lectin complement pathway

Part II: Immunology

Chapter 12

Immune Response

Chapter navigation

- Humoral immune response
- Cell-mediated immune response
- Measurement of immune response

The specific reactivity induced in a host by an antigenic stimulus is known as the **immune response**. In an infectious disease, it is generally equated with protection against invading microorganisms. However, the immune response has a much wider scope and includes reactions against any antigen, living or non-living. It may lead to consequences that are beneficial, unavailing or injurious to the host. It also includes the state of specific non-reactivity (tolerance) induced by certain types of antigenic stimuli.

TYPES OF IMMUNE RESPONSE

An immune response can be of two types: **humoral** (antibody-mediated) and **cellular** (cell-mediated). The two are usually developed together, though at times one or the other may be predominant or exclusive. They usually act in conjunction but sometimes act in opposition.

Types of immunity

• Antibody-mediated immunity (AMI) provides primary defence against most extracellular bacterial pathogens, helps in defence against viruses that infect through the respiratory or intestinal tracts, prevents recurrence of virus infections, and participates in the pathogenesis of immediate (types 1, 2 and 3) hypersensitivity and certain autoimmune diseases.

• Cell-mediated immunity (CMI) protects the host against fungi, viruses and facultative intracellular bacterial pathogens, participates in the rejection of homografts and graft-versus-host reaction, provides immunological surveillance and immunity against cancer, and mediates the pathogenesis of delayed (type 4) hypersensitivity and certain autoimmune diseases.

HUMORAL IMMUNE RESPONSE (ANTIBODY-MEDIATED)

Humoral or antibody-mediated immunity, in response to an antigenic stimulus, occurs in the manner discussed in the following sections.

Production of Antibodies

This consists of three steps:

1. The entry of the antigen, its distribution and fate in the tissues and its contact with appropriate immunocompetent cells (called the **afferent limb**)
2. The processing of the antigen by cells and control of the process of antibody formation (called the **central functions**)
3. The secretion of the antibody, its distribution in tissues and body fluids and the manifestations of its effects (called the **efferent limb**)

Phases

Antibody production follows characteristic phases (Fig. 12.1), which are as follows:

- **Lag phase**, the stage immediately following antigenic stimulus, during which no antibody is detectable in circulation
- **Log phase,** in which the titre of antibodies rises steadily
- **Plateau** or steady state, when there is an equilibrium between antibody synthesis and catabolism
- **Phase of decline,** during which catabolism exceeds production and the titre falls

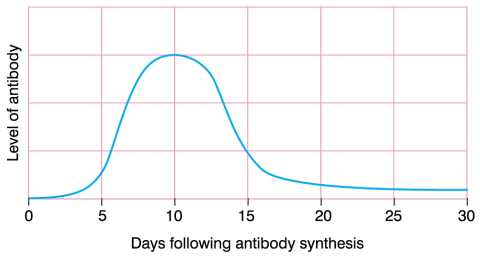


Fig. 12.1 Primary immune response

Primary and secondary responses

Primary response is the antibody response to an initial antigenic stimulus (Fig. 12.1). **Secondary response** is the response to subsequent stimuli with the same antigen.

Primary response differs qualitatively and quantitatively from secondary response (Fig. 12.2). The differences between primary and secondary responses are given in Table 12.1.

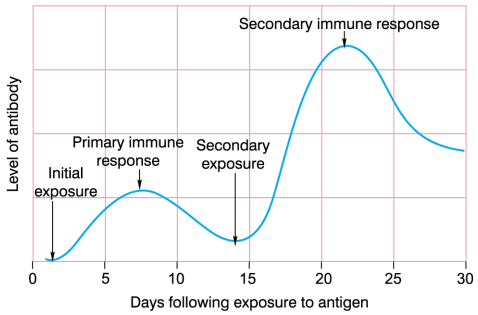


Fig. 12.2 Effect of repeated antigenic stimulus

Table 12.1 Differences between primary and secondary immune response

Features	Primary response	Secondary response
Lag period after immunisation	Usually 4–10 days	Usually 1–3 days
Involving B cell	Naive B cell	Memory B cell
Peak response time	7–10 days	3–5 days
Peak antibody response	Depends on antigen	Usually 100–1000 times more than primary response
Isotype generated	In early response, IgM predominates	IgG predominates
Antigens	Both thymus-dependent and -independent	Only thymus-dependent
Antibody affinity	Lower	Higher
Immunisation needed	Comparatively high dose of antigens, favourably with adjuvants (protein antigens)	Adjuvants not required

The duration of the lag phase and the persistence of the antibody vary with the nature of the antigen. With some antigens such as diphtheria toxoid, the lag phase in the primary response may be as long as 2–3 weeks, while with pneumococcal polysaccharide, antibodies can be detected as early as within a few hours.

Dose

A single injection of an antigen helps in sensitising or priming the immunocompetent cells producing the particular antibody rather than in the actual generation of high levels of antibody. Effective levels of antibody are usually induced only by subsequent injections of the antigen.

It is for this reason that killed vaccines are given in multiple doses for active immunisation. The first injection is known as the **priming** dose and subsequent injections as **booster** doses. With live vaccines, a single dose is sufficient, as the multiplication of the organism in the body provides a continuing antigenic stimulus that acts as both the priming and the booster dose.

When an antigen is injected into an animal already carrying the specific antibody in circulation, a temporary fall in the level of the circulating antibody occurs due to combination of the antigen with the antibody. This has been called the **negative phase**. It is followed by an increase in the titre of the antibody exceeding the initial level.

Appearance of different classes of antibodies from conception to adulthood varies with antigenic stimulation. The general trend of antibody rise and fall is shown in Fig. 12.3.

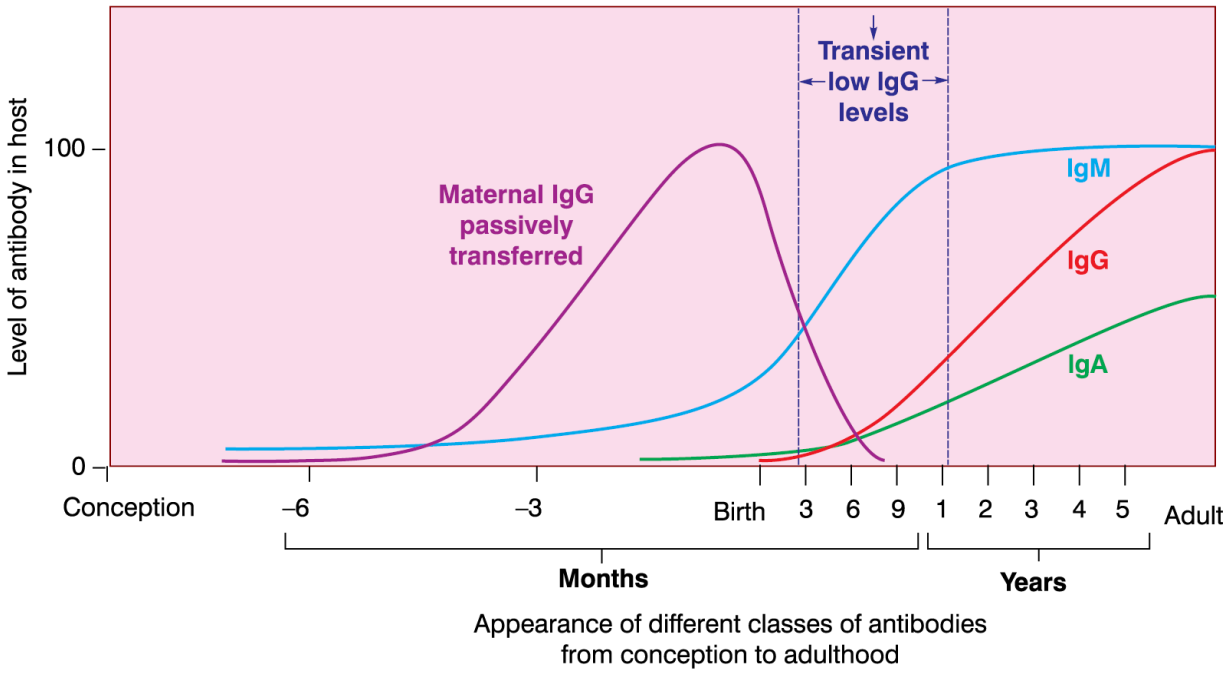


Fig. 12.3 Appearance of different classes of antibodies from conception to adulthood

Antigen Processing and Fate of Antigens Following an Immune Response

Antigens introduced intravenously are rapidly localised in the spleen, liver, bone marrow, kidneys and lungs. Antigen-presenting cells engulf the antigen by **endocytosis**. The endosome fuses with a lysosome, where the antigen is degraded into fragments (e.g., short peptides). These antigenic peptides are then displayed at the surface within a class II histocompatibility molecule. Here, they may be recognised by $CD4^+$ T cells (described below). Antigens that are generated within a cell (e.g., viral proteins in **any** infected cell) are degraded into fragments (e.g., peptides), which involves processing by cytosolic proteasomes. These fragments are displayed on the surface of the cell at the major histocompatibility molecule I, and are recognised by $CD8^+$ T cell.

Removal of particulate antigens

Particulate antigens are removed from circulation in two phases:

- **Non-immune phase** is the first phase during which the antigen is engulfed by the phagocytic cells, broken down and eliminated.

They are broken down by the reticuloendothelial cells and excreted in urine, about 70–80 per cent being thus eliminated within one or two days. In contrast, antigens introduced subcutaneously are mainly localised in the draining lymph nodes, only small amounts being found in the spleen.

- **Immune elimination** begins with the appearance of the specific antibody. In this phase, antigen–antibody complexes are formed and rapidly phagocytosed, resulting in accelerated disappearance of the antigen from circulation.

Removal of soluble antigens

Soluble antigens are removed in three phases:

- Phase of **equilibration** consists of diffusion of the antigen to the extravascular spaces.
- **Metabolic phase** is the phase where the level of the antigen falls due to catabolic decay.
- **Immune elimination** consists of rapid elimination of the antigen with the formation of antigen–antibody complexes. Such complexes may cause tissue damage and may be responsible for 'immune complex diseases' such as serum sickness.

The **rate of elimination** of an antigen is related to the rate at which it is metabolised. Protein antigens are generally eliminated within days or weeks, whereas polysaccharides, which are metabolised slowly, persist for months or years**. Pneumococcal polysaccharide, for instance, may persist for up to 20 years in humans, following a single injection.**

Immune response to an antigen is brought about by three types of cells: (i) antigen-processing cells (**APC**—principally **macrophages** and **dendritic cells**), (ii) **T cells** and (iii) **B cells.**

1. ***Antigen-processing and presentation*** The first step is the capture and processing of antigens by APC and their presentation, in association with the appropriate MHC molecule, to T cells. Only a select subset of hematopoietic lineage cells possesses the specialised property required to efficiently activate or 'prime' an unsensitised T cell,

and thereby initiate a new adaptive immune response. These cells are known as **professional APCs**. On the other hand, antigen presentation by **non-professional APCs** mostly results in T cell tolerance or quiescent memory T cell formation. These cells are lymphatic endothelial cells, neutrophils and mast cells. While T cell activation is essential for most antigens (T cell-dependent antigens such as proteins and erythrocytes), in the case of T cell-independent antigens such as polysaccharides and other structurally simple molecules with repeating epitopes, antibody production does not require T cell participation.

2. ***T and B cell activation*** Only when the processed antigen is presented on the surface of the APC, in association with MHC molecules, to the T cell carrying the receptor (TCR) for the epitope, will the T cell be able to recognise it. In the case of **CD4 (helper T/T_h) cells, the antigen has to be presented complexed with MHC Class II and for CD8 (cytotoxic T/T_c) cells with MHC Class I molecules. B cells,** which possess surface Ig and MHC Class II molecules, can also present antigens to T cells, particularly during the secondary response.

Production of antibodies following an antigenic stimulation

The T_h cell requires two signals for activation. The first signal is a combination of the T cell receptor (TCR) with the MHC Class II-complexed antigen. The second signal is interleukin-1 (IL-1) which is produced by the APC. The activated T_h cell forms interleukin-2 and other cytokines required for B cell stimulation. These include IL-4, IL-5 and IL-6 which act as **B cell activating factor (BCGF)** and the **B cell differentiation factor (BCDF)**. They activate B cells which have combined with their respective antigens to clonally proliferate and differentiate into antibody-secreting plasma cells**. A small proportion of activated B cells, instead of being transformed into plasma cells, become long-living memory cells producing a secondary type of response to subsequent contact with the antigen**.

Cytotoxic T (CD8/TC) cells are activated when they come into contact

with antigens presented along with MHC Class I molecules. They also need a second signal—IL-2, which is secreted by activated T_h cells. On contact with a target cell carrying the antigen on its surface, the activated T_c cells release cytotoxins that destroy the target, which may be virus-infected or tumour cells. Some T_c cells also become memory cells (Fig. 12.4).

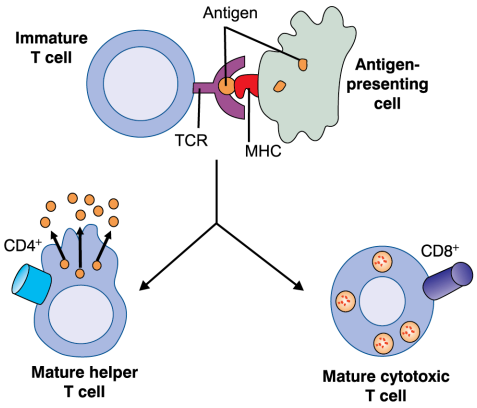


Fig. 12.4 T cell response

Factors influencing antibody production

- ***Genetic factors*** The immune response is under genetic control and differences in immune response to the same antigen shown by different individuals in a species are determined by genetic differences. The terms '**responder**' and '**non-responder**' are used to describe the individual's capacity to respond to a particular antigen. The *Ir* (immune response) genes control this property.
- ***Age*** The embryo is immunologically immature. The capacity to produce antibodies starts only with the development and differentiation of lymphoid organs. During embryonic life, the developing lymphoid cells come into contact with all the tissue antigens of the body released by cellular breakdown, so that all the clones of cells that have specificity towards self-antigens are eliminated.
- ***Immunocompetence*** This is not complete at birth, but continues to develop as the infant grows. The infant has to depend on itself for antibody production from the age of 3–6 months, by which time the maternal antibodies disappear. However, full

competence is acquired only by about 5–7 years for IgG and 10–15 years for IgA.

- ***Nutritional status*** Malnutrition affects immune response adversely, though serum components necessary for immunity are conserved selectively until the nutritional deficiency becomes marked. Protein calorie malnutrition suppresses both humoral and cellular immune response, the latter more severely. Deficiencies of amino acids (tryptophan, phenylalanine, methionine, glycine, isoleucine) and vitamins (vitamin A and B, riboflavin, pyridoxine, pantothenic acid, folic acid) have been shown to cause a decrease in antibody synthesis.
- ***Route of administration*** Humoral immune response is better when the antigens are administered parenterally than through the oral or nasal routes. Large particulate antigens such as bacteria or erythrocytes are more effective when injected into tissues. The route of administration may also influence the type of antibody produced. The oral or nasal route is suitable for the production of IgA antibodies. Inhalation of pollen antigens induces IgE synthesis, whereas the same antigens introduced parenterally lead to the production of IgG antibodies. With some antigens, the route of administration determines whether tolerance or antibody response results. Injection of protein antigens either into the mesenteric vein or intrathymically usually induces tolerance.
- ***Site of administration*** With some antigens, the site of injection seems relevant. The hepatitis B vaccine is less immunogenic following gluteal injection than following injection into the deltoid. This may be due to the paucity of antigen-presenting cells in gluteal fat, delaying the presentation of the antigen to T and B cells.
- ***Size and number of doses*** Antibody response is dependent on the dose of an antigen which is effective only above a minimum critical dose. Further increase in dose (optimal) enhances the intensity of antibody response. However, beyond a certain level, increase in the dose of antigen does not improve the antibody response; instead, such an increase may even inhibit the antibody

response and induce tolerance. This phenomenon is designated as **immunological paralysis** (established by Feltonin in 1949).

- ***Multiple antigens*** When two or more antigens are administered simultaneously, the effects may vary. Antibodies may be produced against the different antigens as though they had been given separately, or antibody response to one or other of the antigens may be enhanced and response to the other may be diminished (antigenic competition).

Antigen Interactions

For optimal effect, the nature and relative proportions of the different antigens in a mixture are carefully adjusted.

- When two bacterial vaccines (for example, typhoid and cholera vaccines) are given in a mixed form, the antibody response to each is not influenced by the other. For example, when toxoids are given along with bacterial vaccines (DPT vaccine) the response to the toxoid is potentiated.
- When diphtheria and tetanus toxoids are given together, with one in excess, the response to the other is inhibited.
- When the triple antigen is given to a person who had earlier received a primary dose of diphtheria toxoid, the response to the tetanus and pertussis antigens will be diminished. Such antigenic competition is important from a practical point of view in immunisation with polyvalent antigens.

Adjuvants

The term **adjuvant refers to any substance that enhances the immunogenicity of an antigen**. Adjuvants may confer immunogenicity on non-antigenic substances, increase the concentration and persistence of the circulating antibody, induce or enhance the degree of cellular immunity and lead to the production of 'adjuvant diseases' such as allergic disseminated encephalomyelitis. A variety of substances exhibit adjuvant activity.

- ***Freund's incomplete adjuvant*** Adjuvants such as **aluminium hydroxide** or **phosphate** and protein antigens incorporated in the water phase of a water-in-oil emulsion delay the release of the antigen from the site of injection and prolong the antigenic stimulus. Others such as **silica particles, beryllium sulphate** and **endotoxins** activate macrophages.
- ***Freund's complete adjuvant*** This constitutes the incomplete adjuvant (as discussed above) in addition to a suspension of killed tubercle bacilli. Besides increasing the humoral immune response, it induces a high degree of cellular immunity (delayed hypersensitivity) as well. However, since it produces a local granuloma, it is unsuitable for human use. The adjuvant effect of the tubercle bacilli is due to a water-soluble peptide MDP (muramyl dipeptide) which induces good antibody response without causing granuloma. Given in mineral oil or as liposomes, it also stimulates cell-mediated immunity. Derivatives of MDP are being developed for human use. Gram-negative bacilli show an adjuvant effect due to their lipopolysaccharide fraction. *Bordetella pertussis*, which has, in addition, a lymphocytosis-promoting factor acting on both T and B cells, acts as a good adjuvant for diphtheria and tetanus toxoid in the triple vaccine. Other adjuvants commonly used with human vaccines are aluminium hydroxide and phosphate.

Immunosuppressive agents

These inhibit immune responses. They are useful in certain situations like transplantation, when it becomes necessary to prevent graft rejection. Examples are as follows:

1. **Radiomimetic drugs** are agents with an action resembling that of X-rays. They belong in general to the class of alkylating agents (e.g., cyclophosphamide and nitrogen mustard). In human beings, cyclophosphamide is given for three days after the antigen completely suppresses the antibody response. It is much less effective when given before the antigen.

2. **Corticosteroids** cause the depletion of lymphocytes from blood and lymphoid organs. They also stabilise the membranes of cells and lysosomes, inhibiting histamine release and the inflammatory response. Therapeutic doses have little effect on antibody formation in human beings. They inhibit the induction and manifestations of delayed hypersensitivity in humans.
3. **Antimetabolites** are substances that interfere with the synthesis of DNA, RNA or both and thus inhibit the cell division and differentiation necessary for humoral and cellular immune response. They include folic acid antagonists (methotrexate), alkylating agents (cyclophosphamide) and analogues of purine (6-mercaptopurine, azathioprine), cytosine (cytosine arabinoside) and uracil (5-fluorouracil). Many antimetabolites find clinical application in the prevention of graft rejection.
4. The drug most widely used now for immunosuppression is **cyclosporine**, a cyclic polypeptide derived from the soil fungus *Tolypocladium inflatum*. It is not cytotoxic for lymphocytes and has no anti-mitotic activity. It selectively inhibits helper T cell activity. A related drug is rapamycin.
5. **Anti-lymphocyte serum (ALS)** is a heterologous antiserum against lymphocytes. The antibody prepared against thymus cells is called **anti-thymocyte serum (ATS)**. The corresponding globulin preparations are called **ALG** and **ATG**. They were used to prevent graft rejection. While all other immunosuppressive agents have undesirable side effects, ALS is devoid of any action other than that on lymphocytes. ALS acts primarily against T lymphocytes and therefore, specifically on cell-mediated immunity. Humoral antibody response to thymus-independent antigens is unaffected and may even be enhanced. ALS acts only against lymphocytes in circulation and not cells in lymphoid organs. As ALS is a foreign protein, its effect is decreased on repeated administration, which may also lead to serum sickness and other hypersensitivity reactions. Monoclonal antibodies against specific lymphocyte membrane antigens have been prepared.
6. **Whole body irradiation** in sub-lethal doses suppresses antibody response. When antigenic stimulus follows 24 hours after irradiation, antibody production does not occur, whereas if the antigen is

administered 2–3 days before irradiation, the antibody response is actually enhanced. The primary response is more radiosensitive than the secondary response.

Immunosuppression by homologous antibody

The humoral immune response to an antigen can be suppressed specifically by passive administration of the homologous antibody. The action appears to be by a feedback mechanism. The primary response is more susceptible to inhibition than the secondary response. The antibody may also combine with the antigen and prevent its availability for the immunocompetent cells.

The inhibitory effect of a passively administered antibody on the humoral immune response has been applied in the prevention of Rh sensitisation in Rh-negative women carrying an Rh-positive fetus. This is achieved by the administration of anti-Rh globulin immediately after delivery (within 72 hours).

This effect is also relevant in the following practices:

- In combined immunisation as in diphtheria and tetanus, the toxoid and antitoxin are given at separate sites. Adsorbed toxoid should be used in this method because the inhibitory effect is much less than with fluid toxoid.
- Intravenous administration of immunoglobulin has been shown to have immunomodulatory effects. It has been used in the treatment of many diseases of presumed immunopathologic origin such as thrombocytopenias and autoimmune hemolytic anemias.

CELLULAR IMMUNE RESPONSE / CELL-MEDIATED IMMUNITY (CMI)

The term cell-mediated immunity (CMI) refers to the **specific immune response mediated by effector T cells generated in response to the antigen**. Both **activated T_h cells** and **cytotoxic T lymphocytes (CTLs)** serve as effector cells in CMI.

Delayed hypersensitivity (DH)
This term refers to the appearance of a skin lesion 48–72 hours after the administration of the antigen. The lesion is an indurated nodule with infiltration by mononuclear cells. DH was found to be

immunologically specific but did not have any relation to antibodies and could not be transferred passively by serum. The cellular basis of DH was shown by Landsteiner and Chase (1942) by its passive transfer in guinea pigs through the injection of leucocytes from sensitised donors.

Scope of Cell-Mediated Immunity (CMI)

Cell-mediated immunity participates in the following immunological functions:

1. ***Delayed hypersensitivity*** Cell-mediated immunity in infectious diseases is evoked by obligate and facultative **intracellular parasites**. These include infections with bacteria (e.g., tuberculosis, leprosy, listeriosis and brucellosis), fungi (e.g., histoplasmosis, coccidioidomycosis and blastomycosis), protozoa (e.g., leishmaniasis and trypanosomiasis) and viruses (e.g., measles and mumps).
2. ***Transplantation immunity and graft-versus-host reaction***
3. ***Immunological surveillance and immunity against cancer***
4. ***Pathogenesis of certain autoimmune diseases*** (e.g., thyroiditis and encephalomyelitis)

Induction of Cell-Mediated Immunity (CMI)

The nature of the antigenic stimulus is important in the induction of CMI. It is best developed following infection with intracellular parasites. Killed vaccines and other non-living antigens do not induce CMI unless administered with the Freund type of adjuvants. **Only T cell–dependent antigens lead to CMI**. The application of certain chemicals on the skin induces DH (Fig. 12.5).

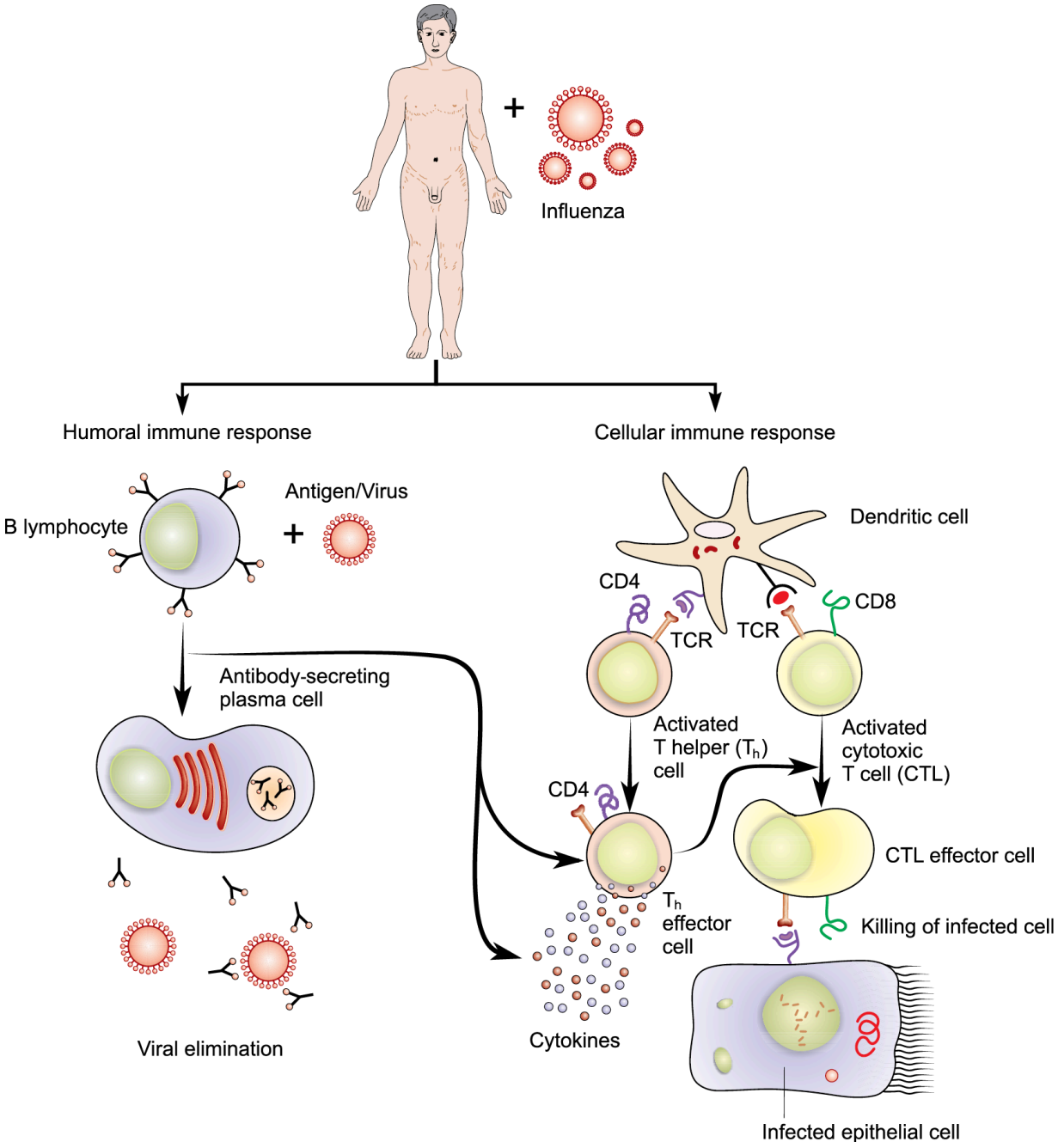
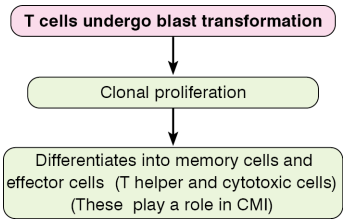


Fig. 12.5 Humoral and cellular immune response

Each T cell bears on its surface a specific receptor (TCR) for one epitope and combines only with antigens carrying that epitope. The chain of events that occur when T cells come in contact with the appropriate antigen are illustrated as follows:


T cells undergo blast transformation
Clonal proliferation
Differentiates into memory cells and effector cells (T helper and cytotoxic cells) (These play a role in CMI)

1. **T cells recognise antigens only when presented with MHC molecules**. Helper T cells react with antigens presented on the surface of macrophages or other cells, **complexed with MHC Class II molecules**. They then release biological mediators (cytokines), which activate macrophages, enabling them to kill intracellular parasites.
2. **Cytotoxic T cells** recognise antigens on the surface of cells (such as virus-infected tumour or allograft cells), and in association with **MHC Class I** molecules, secrete cytokines and destroy the target cells.

Cytokines

Cytokines are biologically active substances released by activated T lymphocytes. They include **interleukin** products of leucocytes, which exert a regulatory influence on other cells**, interferons**, **growth factors** and others with similar effects.

Originally, cytokines were called **lymphokines**. Similar substances produced by monocytes or macrophages were called **monokines.** Initially, they were given names based on their demonstrated biological effect (Table 12.2). Later, they were all grouped under cytokines owing to the fact that most lymphokines exhibit multiple biological effects, the same effect being caused by different lymphokines and due to the lack of precision in their former names.

Table 12.2 Examples of lymphokines

I.	**Affecting macrophages**
	a) Migration inhibitory factor (MIF)
	b) Macrophage activation/aggregation factor (MAF)
	c) Macrophage chemotactic factor (MCF)
II.	**Affecting lymphocytes**
	a) Blastogenic/Mitogenic factor (BF/MF)
	b) T cell growth factor (TGF)

c) B cell growth factor (BGF)

III. Affecting granulocytes

a) Chemotactic factor (CF)

b) Colony-stimulating factor (CSF)

IV. Affecting cultured cells

a) Lymphotoxin (LT)

b) Interferon (IFN)

c) Tumour necrosis factor (TNF)

V. Others

a) Skin-reactive factor (SRF)

b) Transfer factor (TF)

Characteristic features of cytokines

Cytokines are peptide mediators or **intercellular messengers** that regulate immunological, inflammatory and reparative host responses.

They are hormone-like substances, active even at femtomolar (10^{-15} M) concentrations. They differ from endocrine hormones in being produced not by specialised glands but by widely distributed cells (such as lymphocytes, macrophages, platelets and fibroblasts), and by acting not systemically, but locally, near the producing cells (paracrine effect) or directly on the producing cells themselves (autocrine effect).

They are, in general, pleiotropic, having multiple effects on the growth and differentiation of various cell types. There is considerable overlap in the effects of different cytokines. Cloning of cytokines and the availability of monoclonal antibodies against them have helped to characterise them better (Table 12.3).

Table 12.3 Cytokines

Cytokine	Main sources	Major functions
A. INTERLEUKINS		
IL-1 (α and β)	Macrophages and other cell types	Proliferation and differentiation of T, B and other cells; pyrogenic; induce acute phase proteins; bone marrow cell proliferation
IL-2	T cells	Promote growth and differentiation of

		T and B cells, cytotoxicity of T and NK cells, secretion of other lymphokines
IL-3	T cells	Multi-CSF
IL-4	T_h cells	Proliferation of B and cytotoxic T cells; increase IgG and IgE production; enhance MHC Class II and IgE receptors
IL-5	T_h cells	Proliferation of eosinophils, stimulate IgA and IgM production
IL-6	T_h, macrophages, fibroblasts	Promote B cell differentiation; IgG production, acute phase proteins
IL-7	Spleen, bone marrow stromal cells	B and T cell growth factor
IL-8	Macrophages, others	Neutrophil chemotactic factor
IL-9	T cell	T cell growth and proliferation
IL-10	T, B cells, macrophages	Inhibit IFN production and mononuclear cell functions
IL-11	Bone marrow stromal cells	Induce acute phase proteins
IL-12	T cells	Activate NK cells
IL-13	T cells	Inhibit mononuclear cell functions
IL-17	T_h 17 cells	Proinflammatory marker
B. COLONY-STIMULATING FACTORS		
GM-CSF	T cells, macrophages, fibroblasts	T cell and macrophage growth stimulation
G-CSF	Fibroblasts, endothelium	Granulocyte growth stimulation
M-CSF	Fibroblasts, endothelium	Macrophage growth stimulation
C. TUMOUR NECROSIS FACTORS		
TNFα	Macrophages, monocytes	Tumour cytotoxicity, lipolysis, wasting, acute phase proteins, phagocytic cell activation, antiviral and antiparasitic effects, endotoxic shock
TNFβ	T cells	Induce other cytokines
D. INTERFERONS		
IFN-α	Leucocytes	Macrophage activation, augmentation of monocyte and neutrophil function

		and anti-tumour activity
IFN-β	Fibroblasts	Antiviral activity
IFN-γ	T cells	Antiviral, macrophage activation; MHC Class I and II expression on cells
E. OTHERS		
TGF-β	T and B cells	Inhibit T and B cell proliferation and hematopoiesis; promote wound healing
LIF	T cells	Proliferation of stem cells; eosinophil chemotaxis

Important cytokines

Some important cytokines are as follows:

Interleukin-1 IL-1 is a stable polypeptide that retains its activity up to 56°C and between pH 3 and 11. IL-1 occurs in two molecular forms, alpha and beta. It is principally secreted by macrophages and monocytes but can be produced by most other nucleated cells as well. Its production is stimulated by antigens, toxins, injury and inflammatory processes and inhibited by cyclosporin A, corticosteroids and prostaglandins.

The immunological effects of IL-1 include:

- Stimulation of T cells for the production of IL-2 and other lymphokines, B cell proliferation and antibody synthesis, neutrophil chemotaxis and phagocytosis.
- It mediates a wide range of metabolic, physiological, inflammatory and hematological effects by acting on the bone marrow, epithelial and synovial cells, fibroblasts, osteoclasts, hepatocytes, vascular endothelium and other targets.
- IL-1 is an important endogenous pyrogen. Together with the tumour necrosis factor (TNF), it is responsible for many of the hematological changes in septic shock and also enhances the initial meningeal inflammation in bacterial meningitis.
- Cytokine inhibitors such as dexamethasone have been found to be protective against the sequelae of such excessive meningeal inflammation.

- IL-1 has a beneficial effect in severe infections in immunocompromised hosts.

Interleukin-2 This is the T cell growth factor (TCGF) produced by activated T cells.

- It induces T cell proliferation, enables their maintenance in continuous culture and contributes greatly to the understanding of T cell function.
- It is a powerful modulator of the immune response. It is the major activator of T and B cells and stimulates cytotoxic T cells and NK cells.
- It converts some null cells (LGL) into lymphokine- activated killer (LAK) cells which can destroy NK-resistant tumour cells. This property has been used in the treatment of certain types of cancer.

Interleukin-3 IL-3 is a growth factor for bone marrow stem cells.

- It stimulates multilineage hematopoiesis and is therefore also known as the multi-colony-stimulating factor (multi-CSF).

Interleukin-4 IL-4 was formerly known as the B cell growth factor-1 (BCGF-1).

- It activates resting B cells and acts as a B cell differentiating factor.
- It acts as a growth factor for T cells and mast cells. It enhances the action of cytotoxic T cells.
- It may have a role in atopic hypersensitivity as it augments IgE synthesis.

Interleukin-5 IL-5 was formerly known as the B cell growth factor-II (BCGF-2).

- It causes proliferation of activated B cells.

- It induces maturation of eosinophils.

Interleukin-6 IL-6 is produced by stimulated T and B cells, macrophages and fibroblasts.

- It induces immunoglobulin synthesis by activated B cells and formation of IL-2 receptors on T cells.
- It has a stimulatory effect on hepatocytes, nerve cells and hematopoietic cells.
- It acts as an inflammatory response mediator in host defence against infections.

Colony-stimulating factor (CSF) These cytokines stimulate the growth and differentiation of pluripotent stem cells in the bone marrow. They have been named after the types of cell colonies they induce in soft agar culture, for example, granulocyte CSF (G-CSF) or mononuclear CSF (M-CSF).

- IL-3, which induces growth of all types of hematopoietic cells, is known as multi-CSF. In the body, it may cause other effects also, presumably by inducing cascades of other cytokines.
- CSFs are responsible for adjusting the rate of production of blood cells according to requirements, for example, the massive granulocyte response seen in pyogenic infections. They have clinical applications for treating hematopoietic dysfunctions in infections and malignancies.

Tumour necrosis factor (TNF) The tumour necrosis factor occurs as two types, alpha and beta.

- It induces hemorrhagic necrosis in certain tumours, based on which, it has been named. **It was originally described as cachectin, a serum factor causing the wasting syndrome (cachexia) during chronic infections**. This has been renamed TNFα. It is formed principally by activated macrophages and monocytes. It resembles IL-1 in possessing a very wide spectrum of biological activities such as:

- Participation in the manifestations of endotoxic shock.
- Exertion of an immunomodulatory influence on other cytokines. TNFα, formerly known as lymphotoxin, is produced principally by T helper cells. Its effects are similar to those of TNFα.

Interferons (IFN) Originally identified as antiviral agents, interferons are now classified as cytokines. There are **three classes of IFNs**:

1. IFN-α produced by leucocytes,
2. IFN-β produced by fibroblasts and
3. IFN-γ by T cells activated by antigens, mitogens or exposure to IL-2.

IFN-α causes many immunological effects such as:

- Macrophage activation
- Augmentation of neutrophil and monocyte function
- Anti-tumour activity

Other cytokines The transforming growth factor beta (TGF-β) was so named because of its ability to transform fibroblasts. Besides acting as a growth factor for fibroblasts and promoting wound healing, it also acts as a down-regulator of some immunological and hematological processes.

The leukemia inhibitory factor (LIF) produced by T cells helps stem cell proliferation and eosinophil chemotaxis.

Regulation of cytokine production

Cytokine production is regulated by exogenous stimuli such as antigens and mitogens, as well as by endogenous factors such as neuroendocrine hormonal peptides (corticosteroids and endorphins) and products of lipoxygenase and cyclooxygenase pathways. They **also regulate each other by positive and negative feedback.** A number of cytokines (e.g., IL-1, IL-2, IL-3, colony-stimulating factors and interferons) have already found therapeutic application. IL-17 is a proinflammatory cytokine that plays an important role in autoimmune disorders. **With a better understanding of their properties, it is possible that many cytokines, their agonists and antagonists could eventually be used in the management of inflammatory, infectious, autoimmune and neoplastic**

conditions.

Detection of Cell-Mediated Immunity (CMI)

The original method for detecting CMI was the skin test for delayed hypersensitivity (e.g., the tuberculin test). A number of in vitro correlates of CMI have now become available. These include the following:

- Lymphocyte transformation test—transformation of cultured sensitised T lymphocytes on contact with the antigen).
- Target cell destruction—killing of cultured cells by T lymphocytes sensitised against them).
- Migration inhibitory factor test, which is commonly used. As originally described, this consisted of incubating in a culture chamber, packed peritoneal macrophages in a capillary tube. The macrophages migrate to form a lacy, fan-like pattern. If the macrophages are from a guinea pig sensitised to tuberculoprotein, addition of tuberculin to the culture chamber will inhibit the migration (Fig. 12.6).

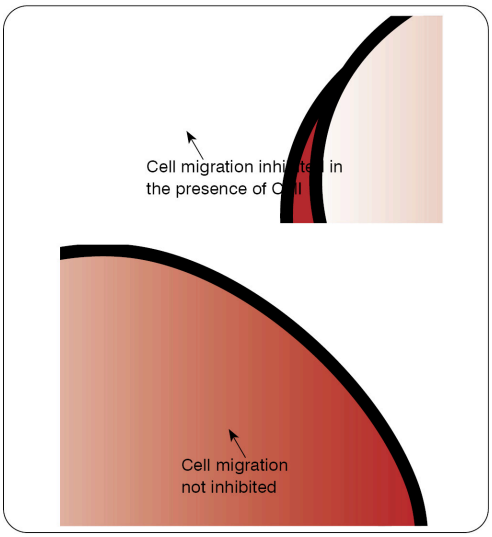


Fig. 12.6 Inhibition of migration of macrophage cells

Migration inhibitory factor test has been adapted for clinical use by incubating human peripheral leucocytes in capillary tubes in culture chambers. When an antigen to which the individual has CMI is introduced into the culture medium, the leucocytes are prevented from migrating. By comparison with the control, it is possible to make a semi-quantitative assessment of the migration inhibition.

Transfer Factor (TF)

Passive transfer of CMI was first achieved (by Lawrence in 1954) by the injection of viable leucocytes from sensitised donors. The leucocyte extract is known as the 'transfer factor' (TF). The transferred immunity is specific in that CMI can be transferred only to those antigens to which the donor is sensitive.

- TF is a dialysable, low-molecular-weight substance (MW 2,000–4,000), resistant to trypsin, DNase, RNase and freeze-thawing. It is stable for several years at –20°C and in the lyophilised form at 4°C. It is inactivated at 56°C in 30 minutes. It is not antigenic.
- Chemically, it appears to be a polypeptide–polynucleotide.
- TF is highly potent—an extract from 0.1 mL of packed leucocytes is sufficient for transfer. The transferred CMI is systemic and not local at the injected site. Following TF injection, DH and various in vitro correlates of CMI can be demonstrated in the recipient. **Humoral immunity is not transmitted by TF.**
- TF transfers CMI to all the antigens to which the donor is sensitive as a whole. It is possible to transfer CMI from the recipient to another in a serial fashion.

The mechanism of action of TF is not known. TF could be an informational molecule or a specific gene derepressor capable of inducing antigenically uncommitted lymphocytes to produce antigen-specific receptors. TF activity was, until recently, demonstrable only in humans but it has now been reported in monkeys, guinea pigs and mice.

Applications of transfer factor in clinical practice

- Used to restore immune capacity in patients with T cell deficiency (Wiskott–Aldrich syndrome)
- Used in the treatment of disseminated infections associated with deficient CMI (e.g., lepromatous leprosy, tuberculosis and mucocutaneous candidosis)
- Employed in the treatment of malignant melanoma and may be beneficial in other types of cancer

- Use has been suggested in some autoimmune diseases (like systemic lupus erythematosus and rheumatoid arthritis) and diseases of unknown origin (like sarcoidosis and multiple sclerosis)

IMMUNOLOGICAL TOLERANCE

Immunological tolerance or immunological unresponsiveness is a condition in which contact with an antigen specifically abolishes the capacity to mount an immune response against that particular antigen when it is administered subsequently. This non-reactivity is specific to the particular antigen; immune reactivity to other antigens remains unaffected.

Burnet and Fenner (1949) suggested that an antigen that comes into contact with the immunological system during embryonic life would be recognised as a self-antigen and would not induce any immune response. They postulated that tolerance could be induced against foreign antigens if they were administered during embryonic life. This was proved experimentally by Medawar and his colleagues (1953) using two strains of syngeneic mice.

Specific immunological tolerance When a skin graft from one inbred strain of mice (CBA) is applied on a mouse of another strain (A), it is rejected. If CBA cells are injected into fetal or newborn strain A mice, however, the latter will freely accept skin grafts from CBA mice when they grow up. The content of the self-antigen appears to be enlarged by contact with a foreign antigen during embryonic life. Development of tolerance is not confined to the embryo or newborn but can also occur in adults. Tolerance may be total or partial, short-lived or long-lasting.

Factors Affecting Immunological Tolerance

Factors affecting the induction, degree and duration of tolerance are as follows:

- ***Species and immunocompetence of the host*** Strain differences in tolerance induction are seen within species (e.g., rabbits and mice can be rendered tolerant more rapidly than guinea pigs and chickens). The higher the degree of immunocompetence of the

host, the more difficult it is to induce tolerance. It is for this reason that embryos and newborns are particularly susceptible to the induction of tolerance. **Tolerance can be induced in adults in whom immunocompetence is temporarily interrupted by immunosuppressive agents**. Induction of tolerance is very difficult in adults already immunised against the antigen.

- ***Nature of antigen*** The physical state of the antigen is important. Soluble antigens and haptens are more tolerogenic than particulate antigens. The tolerogenicity of an antigen can be modified by certain procedures. When human gamma globulin is heat-aggregated, it is highly immunogenic in mice but is tolerogenic when disaggregated. Solutions of serum proteins centrifuged at high speed separate into tolerogenic supernatant and immunogenic sediment fractions.
- ***Dose of the antigen*** The induction of tolerance is dose-dependent. There is a threshold dose, below which tolerance is not induced. Further increase in dose increases the duration of tolerance. With certain antigens, tolerance can be induced by two types of doses, one high and the other low, with intermediate doses producing immunity instead of tolerance. These are known as '**high zone**' and '**low zone**' tolerance, respectively. A special type of high zone tolerance is **Felton's immunological paralysis**. The duration of tolerance is variable. Tolerance can be prolonged by repeated tolerogenic stimuli.
- ***Route of administration*** The best route to induce tolerance is the intradermal route, whereby the antigen equilibrates throughout the extra- and intravascular compartments. For antigens that do not equilibrate readily or that are rapidly eliminated, the route of choice is intravenous. Certain haptens that are immunogenic in guinea pigs by the intradermal route are tolerogenic orally or intravenously.

Naturally occurring tolerance is found in certain viral infections such as **congenital rubella** and **cytomegalovirus** infections in which there is persistent viremia with decreased ability in the production of neutralising antibodies (persistent tolerant infection).

In **lymphocytic choriomeningitis** infection in carrier mice, the virus may persist in virtually all the cells and tissues and be transmitted vertically to the offspring without any demonstrable immune response or pathogenic effect. When the tolerance is interrupted by an induction of antibody or an injection of sensitised lymphocytes, disease results.

Tolerance can be overcome spontaneously or by an injection of cross-reacting immunogens. For example, tolerance to bovine serum albumin in rabbits can be abolished by immunisation with cross-reacting human serum albumin. In general, tolerance to living agents is more lasting than that to non-living substances.

Mechanism of Immunological Tolerance

The mechanism of tolerance is not clear. The more likely mechanism may be the elimination of T_H cells, which effectively prevents B cell activation. This is the '**central mechanism**' of tolerance induction. In other instances, the mechanism may be an '**afferent block**' in which access of the antigen to immunocompetent cells is interfered with, or an '**efferent block**' in which the antibody synthesised is neutralised or destroyed. T and B lymphocytes appear to possess differing sensitivity to tolerance induction, the former being more susceptible. In general, high doses of antigens induce B cell tolerance and repeated minute doses of antigens induce T cell tolerance.

Tolerance to humoral and cellular types of immunity is usually induced simultaneously. '**Split tolerance**' can also occur where unresponsiveness is established for one parameter of the immune response and not for the other. In guinea pigs, DH to tuberculin can be inhibited without affecting the production of a circulating antibody by the injection of tuberculoprotein prior to immunisation with BCG.

THEORIES OF IMMUNE RESPONSE

A succession of theories have been put forward from time to time to explain the versatility, specificity, memory and other features of immune response. Theories of immunity fall into two categories: instructive and selective.

- **Instructive theories** postulate that an immunocompetent cell is capable of synthesising antibodies of any specificity. The antigen

encounters an immunocompetent cell and instructs it to produce the complementary antibody.

- **Selective theories**, on the contrary, shift the emphasis from the antigen to the immunocompetent cell. They postulate that immunocompetent cells have a restricted immunological range. The antigen exerts only a selective influence by stimulating the appropriate immunocompetent cell to synthesise an antibody.

Clonal Selection Theory

With the advent of cellular and molecular mechanisms, clonal selection has come to be the most relevant and accepted theory. This theory, which shifted immunological specificity to the cellular level, was proposed by Burnet (1957).

According to the clonal selection hypothesis, during immunological development, cells capable of reacting with different antigens were said to be formed by a process of somatic mutation. Clones of cells that had immunological reactivity with self-antigens were said to be eliminated during embryonic life. Such clones are called **forbidden clones**. The result of the contact with the specific antigen was cellular proliferation to form clones synthesising the antibody.

The clonal selection theory is more widely accepted than other theories, though it is unable to account for all the features of immune response. A variety of modifications and alternative theories have been proposed in recent times but none have succeeded in explaining all that is known of immunity.

Abandoned Theories

Some of the theories which were abandoned are as follows:

Side chain theory This, the first plausible theory of immune response, was proposed by Ehrlich (1900). Cells were considered to have surface 'receptors' capable of reacting with substances having complementary 'side chains'. The theory postulated that these receptors anchor nutrients to cells before assimilation and that when foreign antigens are introduced, they combine with cell receptors that have a complementary fit. This was said to inactivate the receptors and interfere with the absorption of nutrients. As a

compensatory mechanism, there was said to be an overproduction of the same type of receptors which then circulate in blood as antibodies.

While it explains the specificity of antibody response, this theory had to be abandoned when Landsteiner demonstrated that antibodies could be formed not only against natural antigens but also against various synthetic chemicals.

Direct template theories Instructive theories were proposed by Breinl and Haurowitz (1930), Alexander (1931) and Mudd (1932). According to these, the antigen (or antigenic determinant) enters antibody-forming cells and serves as a 'template' against which antibody molecules are synthesised so that they have combining sites complementary to the antigenic determinant. Pauling (1940) presented a more detailed model suggesting that specificity was determined by the folding of the antibody polypeptide chains to form a tertiary structure fitting the antigenic determinant.

Indirect template theory According to Burnet and Fenner (1949), the entry of the antigenic determinant into the antibody-producing cell induced in it a heritable change. Thus, a 'genocopy' of the antigenic determinant was said to be incorporated in the genome of the cell and transmitted to the progeny cells (indirect template). This theory explained specificity and secondary response but became untenable with advances in the molecular biology of protein synthesis.

Natural selection theory In 1955, Jerne postulated that about a million globulin (antibody) molecules are formed in embryonic life, which cover the full range of antigenic specificities. These globulins were said to be the 'natural antibodies'. It was postulated that when an antigen is introduced, it combines selectively with the globulin that has the nearest complementary 'fit'. This complex was then said to 'home' in on the antibody-forming cells and stimulate them to synthesise the same kind of antibody. However, this theory did not explain the fact that immunological memory resides in cells and not in serum.

IDIOTYPES

The distinct amino acid sequences at the antigen-combining site and the adjacent parts of the variable region are termed **idiotypes**. The idiotype can, in turn, act as an antigenic determinant and induce anti-idiotypic antibodies.

These, in turn, can induce antibodies to them and so on, forming an idiotype network which is postulated to regulate the amount of antibodies produced and the number of antibody-forming cells in action. The variable region of an immunoglobulin molecule carrying the antigen-combining site is different in different antibodies. For his theoretical contribution to antibody formation and regulation of the immune system by network hypothesis, Niels K. Jerne was awarded the Nobel Prize for Medicine in 1984.

The genetic basis of antibody diversity has been clarified recently. An individual has the capacity to produce an estimated 10^8 different antibody molecules. To have each such antibody molecule coded for by a separate gene would require millions of genes to be set apart for antibody production alone. The discovery of **split genes for immunoglobulins** demolished the longstanding dogma of 'one–gene–one protein' and has important implications in biology that extend beyond immunology alone. For this discovery, Susumu Tonegawa was awarded the Nobel Prize for Medicine in 1987.

Key points

- Two phases are seen in the response of the immune system to an antigen: primary and secondary.
- Primary immune response: It is the immune response to a microorganism (or antigen) that is encountered for the first time; the latent period is usually long; response itself may be transient and mild.
- Secondary immune response: It is the immune response that is elicited when the immune system encounters the same microorganism (antigen) on a second or subsequent occasion; response is more rapid (short latent period), vigorous and prolonged.
- The lymphocytes involved in the specific immune response are of two principal types: B lymphocytes (B cells) and T lymphocytes (T cells).
- B lymphocytes are precursors of the antibody-producing cells (plasma cells). The immune response can be of two types—humoral (antibody-mediated) and cellular (cell-mediated).
- The antibodies produced by a single clone and directed against a single antigenic determinant are called monoclonal antibodies. They are very useful tools for diagnostic and research techniques.
- T lymphocytes are responsible for cell-mediated immunity. When a T cell encounters an antigen recognised by the T cell receptors, a number of substances, collectively called lymphokines, are released; these activate macrophages and increase their ability to destroy the pathogens.

- Immunological tolerance or unresponsiveness is a condition in which contact with an antigen specifically abolishes the capacity to mount an immune response against that particular antigen when it is administered subsequently.
- Theories of immunity fall into two categories: instructive and selective. The instructive theories postulate that an immunocompetent cell is capable of synthesising antibodies of any specificity. Selective theories shift the emphasis from the antigen to the immunocompetent cell. They postulate that immunocompetent cells have a restricted immunological range.

Questions

Essays

1. Describe the principle and method of preparation of monoclonal antibodies in the laboratory.
2. Briefly describe types of immune responses and their functions.

Short Answers

1. Humoral immunity
2. Cell-mediated immunity
3. Primary and secondary response
4. Theories of immune response
5. Cytokines
6. Transfer factor
7. Immunosuppressive agents
8. Immunological tolerance
9. Differences between primary and secondary response
10. Applications of monoclonal antibodies in clinical microbiology
11. Factors that influence antibody production

Part II: Immunology

CHAPTER 13

Antigen–Antibody Reactions

Chapter navigation

- Precipitation
- Agglutination
- Complement fixation
- Neutralisation
- Radioimmunoassays
- Enzyme immunoassays
- Chemiluminescence immunoassay
- Immunoelectroblot/Western blot techniques
- Immunofluorescent assays
- Immunoelectron assays
- Immunochromatographic assays

Antigens and antibodies combine with each other specifically and in an observable manner. The binding occurs between the antigenic epitope and the variable domain of the antibody. This exclusive reaction has led to the development of several immunological assays to detect infectious diseases, monitor humoral immunity and detect molecules of medical interest.

STAGES IN ANTIGEN–ANTIBODY REACTIONS

The reaction occurs in three different stages.

1. ***Primary stage*** This is the initial interaction between antigens and antibodies, **without any visible effects**. This reaction is:

   - Rapid, occurs even at low temperatures and is reversible
   - Based on the general laws of physical chemistry and thermodynamics
   - A combination of intermolecular forces such as Van der Waal's forces (ionic bonds and hydrogen bonding, rather than the firmer covalent bonding)

- Detectable by estimating free and bound antigens or antibodies separately in the reaction mixture by a number of physical and chemical methods, e.g., by the use of: a) markers such as radioactive isotopes, fluorescent dyes or ferritin or b) enzyme-linked immune assays.

2. ***Secondary stage*** In most, but not all, instances, the primary stage is followed by the secondary stage, leading to **demonstrable events** such as:

   - Precipitation
   - Agglutination
   - Lysis of cells
   - Killing of live antigens
   - Neutralisation of toxins and other biologically active antigens
   - Fixation of complement
   - Immobilisation of motile organisms

   - Enhancement of phagocytosis

An antibody that causes agglutination is called **agglutinin** while one that causes precipitation is called **precipitin**; the corresponding antigens are called **agglutinogen** and **precipitinogen** respectively. A single antibody can cause precipitation, agglutination and most other serological reactions and an antigen can stimulate the production of different classes of immunoglobulins which differ in their reaction capacities as well as in other properties (Table 13.1).

3. ***Tertiary stage*** Some antigen–antibody reactions occurring in vivo **initiate chain reactions** that lead to the neutralisation or destruction of injurious antigens or tissue damage. These are tertiary reactions and include humoral immunity as well as clinical allergy and other immunological diseases.

Antigen and antibody bind by non-covalent bonds such as:

- **Electrostatic forces** Attraction between opposite charges.

- **Hydrogen bonds** Hydrogen is shared between two electronegative atoms.
- **Van der Waal's forces** Interaction between outer electron clouds of two or more electrons.
- **Hydrophobic forces** Forcing together of hydrophobic groups by water.

Table 13.1 Comparative efficiency of the immunoglobulin classes in different serological reactions

Reaction	*IgG*	*IgM*	*IgA*
Precipitation	Strong	Weak	Variable
Agglutination	Weak	Strong	Moderate
Complement fixation	Strong	Weak	Negative
Lysis	Weak	Strong	Negative

USES OF ANTIGEN–ANTIBODY REACTIONS

- In the body, they form the **basis of antibody-mediated immunity** in infectious diseases, or of tissue injury in some types of hypersensitivity and autoimmune diseases.
- In the laboratory, they help in the:
  - **Diagnosis of infections** by detecting antibodies or antigens.
  - **Detection and quantitation** of either antigens or antibodies.
- In **sero-epidemiological surveys**, they aid in screening a population for the presence of the antibodies for a particular infection.

GENERAL FEATURES OF ANTIGEN–ANTIBODY REACTIONS

1. The **reaction is specific**, an antigen combining only with its homologous antibody and vice versa. The specificity, however, is not

absolute and 'cross-reactions' may occur due to antigenic similarity or relatedness.

2. **Entire molecules**, and not fragments, are involved in these reactions. When an antigenic determinant present in a large molecule or on a 'carrier' particle reacts with its antibody, whole molecules or particles are agglutinated.
3. There is **no denaturation of the antigen or the antibody** during the reaction.
4. The **combination occurs at the surface**. Therefore, it is the surface antigens that are immunologically relevant. Antibodies to the surface antigens of infectious agents are generally protective.
5. The **combination is firm but reversible.** The firmness of the union is influenced by the affinity and avidity of the reaction.

Affinity refers to the intensity or strength of binding between the epitope of the antigen and antibody molecules. Affinity is a quantitative measure of the binding strength between an antibody and an epitope. Low-affinity antibodies bind antigens weakly and tend to dissociate readily, whereas high-affinity antibodies bind antigens more tightly and remain bound longer.

Avidity is the strength of the bond after the formation of the antigen and multivalent antibody complexes. It is a better measure of its binding capacity within biological systems (e.g., reaction of an antibody with antigenic determinants on a virus or bacterial cell). Pentameric IgM often has lower affinity than IgG but the high avidity of IgM, resulting from its higher valence, enables it to bind antigens effectively.

6. Both antigens and antibodies participate in the formation of agglutinates or precipitates.
7. Antigens and antibodies can combine in varying proportions, unlike chemicals with fixed valencies. Both antigens and antibodies are multivalent. Antibodies are generally bivalent, though IgM molecules may have five or ten combining sites. Antigens may have valencies up to the hundreds.

Parameters of serological tests

Antigen–antibody reactions in vitro are known as serological reactions. The parameters of serological tests are the following:

Sensitivity refers to the ability of the test to detect even very minute quantities of antigen or antibody. When a test is highly sensitive, false negative results are absent or minimal.

Specificity refers to the ability of the test to detect reactions between homologous antigens and

antibodies only, and with no other. In a highly specific test, false positive reactions are absent or minimal, and only the true positive reactions are detected.
In general, sensitivity and specificity are inversely proportional.
Qualitative assays refer to the mere detection of the presence of an antigen or antibody (e.g., the test is reactive or non-reactive).
Quantitative assays refer to the estimation of the quantity of an antigen or antibody (the simplest is by serially diluting the sample and detecting the antigen or antibody present in the highest dilution).There are other sensitive methods that estimate the exact quantity in micrograms or nanograms.

REACTIONS ON WHICH ANTIGEN–ANTIBODY ASSAYS ARE BASED

Some antigen–antibody reactions used in the laboratory are based on the following reactions:

A. PRECIPITATION REACTION

1. ***Precipitation*** When a **soluble** antigen combines with its antibody in the presence of electrolytes (NaCl) at a suitable temperature and pH, the antigen–antibody complex forms an insoluble precipitate.
2. ***Flocculation*** When, instead of sedimenting, the precipitate remains suspended as floccules, the reaction is known as **flocculation**.

Precipitation can take place in liquid media or in gels such as agar, agarose or polyacrylamide.

Phases

The amount of precipitate formed is influenced by the relative proportions of antigens and antibodies. If increasing quantities of antigens are added to the same amount of antiserum in different tubes, precipitation occurs most abundantly in one of the middle tubes in which the antigen and antibody proportions are optimal or equivalent. In the preceding tubes, in which the antibody is in excess and in the later tubes, in which the antigen is in excess, there is weak or even absence of precipitation.
If the amounts of precipitate in the different tubes are plotted on a graph, the resulting curve will have three phases:

- ***Prozone phenomenon*** This is caused by excess antibody in the test system. Failure of a visible reaction is due to the inhibition of

lattice formation by the excess antibody.

- ***Zone of equivalence*** Here, the antigen and antibody are in optimum proportions. Lattice formation and visible reactions are enhanced.
- ***Post-zone phenomenon*** This is caused by the presence of excess antigen in the test system. No visible reaction occurs.

The prozone and post-zone phenomena may be corrected by making serial dilutions of serum, thereby reducing the concentration of antigen or antibody in the test serum, and optimising the concentrations of the antigen and antibody.

The prozone is of importance in clinical serology, as sera rich in antibody may sometimes give a false negative precipitation or agglutination result, unless several dilutions are tested. A classic example is the Brucella agglutination test.

Mechanism

Precipitation results when a large lattice is formed, consisting of alternating antigen and antibody molecules. This is possible only in the zone of equivalence. In the zones of antigen or antibody excess, the lattice does not enlarge, as the valencies of the antibody and the antigen, respectively, are fully satisfied (Fig. 13.1). The lattice hypothesis holds good for agglutination also, where each antibody must have at least two binding sites and the antigen must be multivalent.

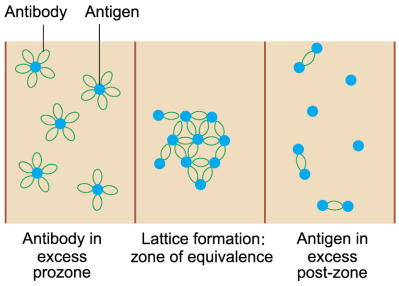


Fig. 13.1 Lattice hypothesis

Applications of precipitation reactions

The precipitation test may be carried out as a qualitative or quantitative test. It is sensitive in the detection of antigens, and as little as 1 mg of protein can be detected. It is relatively less sensitive for the detection

of antibodies. Precipitation tests have several applications:
- Grouping of streptococci by the Lancefield technique
- The VDRL test for syphilis
- To standardise toxins and toxoids
- To test toxigenicity in diphtheria bacilli
- Forensic application in the identification of blood and seminal stains
- Testing for food adulterants

Types

The following types of precipitation and flocculation tests are in common use:

Immunodiffusion (precipitation in gel)

There are several advantages in allowing precipitation to occur in a gel rather than in a liquid medium. The reaction is visible as a distinct band of precipitation, which is stable and can be stained for preservation, if necessary. As each antigen–antibody reaction gives rise to a line of precipitation, the number of different antigens in the reacting mixture can be readily observed. Immunodiffusion also indicates identity, cross-reaction and non-identity between different antigens. Immunodiffusion is usually performed in a soft (1%) agar or agarose gel.

Modifications of the immunodiffusion test

- ***Single diffusion in one dimension (Oudin procedure)*** The antibody is incorporated in agar gel in a test tube and the antigen solution is layered over it. The antigen diffuses downward through the agar gel, forming a line of precipitation.
- ***Double diffusion in one dimension (Oakley–Fulthorpe procedure)*** Here, the antibody is incorporated in gel, above which is placed a column of plain agar. The antigen is layered on top of this. The antigen and antibody move towards each other through the intervening column of plain agar and form a band of precipitate at the point where they meet at optimum proportion (Fig. 13.2a).
- ***Single diffusion in two dimensions (radial immunodiffusion)*** Here, the antiserum is incorporated in agar gel poured on a flat surface (slide or petri dish). The antigen is

added to the wells cut on the surface of the gel. It diffuses radially from the well and forms ring-shaped bands of precipitation (halos) concentrically around the well. The diameter of the halo gives an estimate of the concentration of the antigen. This is used for the estimation of the immunoglobulin classes in sera (Fig. 13.2b).

- ***Double diffusion in two dimensions (Ouchterlony procedure)*** This method is most widely employed and helps to compare different antigens and antisera directly. Agar gel is poured on a slide and wells are cut using a template. The antiserum is placed in the central well, and different antigens in the surrounding wells. If two adjacent antigens are identical, the lines of precipitate formed by them will fuse. If they are unrelated, the lines will cross each other. Cross-reaction or partial identity is indicated by spur formation. A special variety of double diffusion in two dimensions is the **Elek test for toxigenicity in diphtheria bacilli**. When diphtheria bacilli are streaked at right angles to a filter paper strip carrying the antitoxin, implanted on a plate of suitable medium, arrowhead-shaped lines of precipitation appear on incubation if the bacillus is toxigenic (Fig. 13.2c).

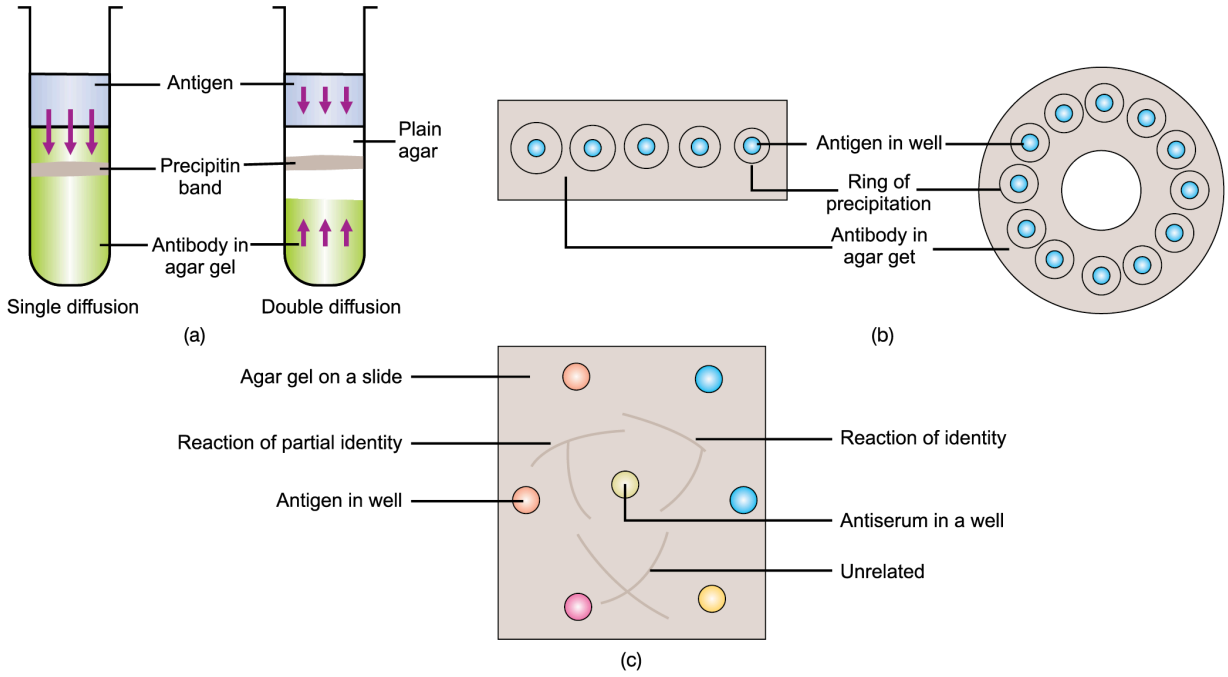


Fig. 13.2 (a) Single and double diffusion in one dimension; (b) Single diffusion in two dimensions; (c) Double diffusion in two dimensions

- ***Immunoelectrophoresis*** This involves the electrophoretic separation of a composite antigen (such as serum) into its constituent proteins, followed by immunodiffusion against its antiserum, resulting in separate precipitin lines, indicating reaction between each individual protein and its antibody. The technique is performed on agar or agarose gel on a slide, with an antigen well and an antibody trough cut into it. The test serum is placed in the antigen well and electrophoresed for about an hour. Antibody against human serum is then placed in the trough and diffusion allowed to proceed for 18–24 hours. The resulting precipitin lines can be photographed and the slides dried, stained and preserved for the record. Over 30 different proteins can be identified by this method in human serum. This is **useful for testing for normal and abnormal proteins in serum and urine** (Fig. 13.3).

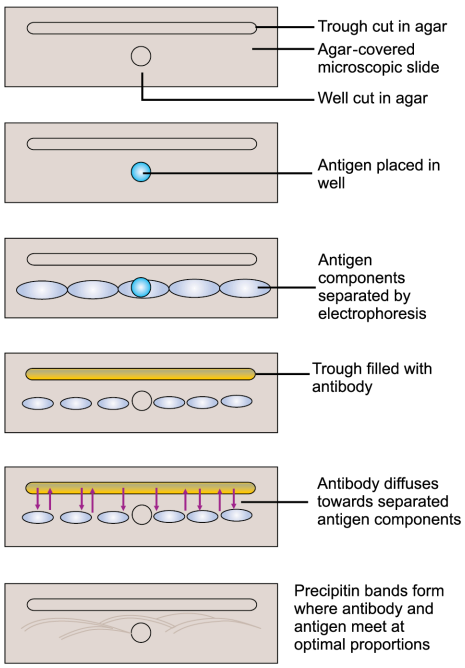

Trough cut in agar
Agar-covered microscopic slide
Well cut in agar
Antigen placed in well
Antigen components separated by electrophoresis
Trough filled with antibody
Antibody diffuses towards separated antigen components
Precipitin bands form where antibody and antigen meet at optimal proportions

Fig. 13.3 Immunoelectrophoresis

- ***Electroimmunodiffusion*** The development of precipitin lines can be speeded up by electrically driving the antigen and antibody with diffusion using various methods in the clinical laboratory as given below.
    - ***Counterimmunoelectrophoresis (CIE, counter-current immunoelectrophoresis)*** This involves simultaneous electrophoresis of the antigen and antibody in gel in opposite directions, resulting in precipitation at a point between them (Fig. 13.4). This method produces visible precipitation lines within 30 minutes and is ten times more sensitive than the standard double diffusion techniques. The clinical applications comprise detecting various antigens such as alpha-fetoprotein in serum and specific antigens of cryptococcus and meningococcus in cerebrospinal fluid (advanced rapid immunological techniques have replaced these methods).

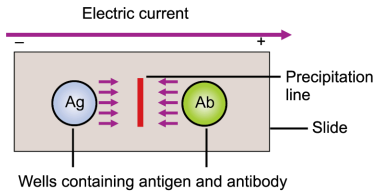


Fig. 13.4 Counterimmunoelectrophoresis

- ***One-dimensional single electroimmunodiffusion (rocket electrophoresis)*** The main application of this technique is for quantitative estimation of antigens. The antiserum to the antigen to be quantitated is incorporated in agarose and gelled on the glass slide. The antigen, in increasing concentrations, is placed in wells punched in the set gel. The antigen is then electrophoresed into the antibody-containing agarose (Fig. 13.5). The pattern of immunoprecipitation resembles a rocket (hence, the name).

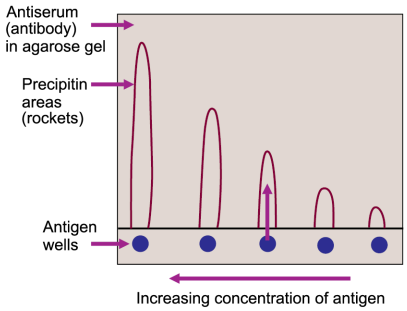


Fig. 13.5 Rocket electrophoresis

- ***Two-dimensional electrophoresis*** In Laurell's two-dimensional electrophoresis, the antigen mixture is first electrophoretically separated in a direction perpendicular to that of the final rocket stage. By this method, each of several antigens in a mixture can be quantitated (Fig. 13.6).

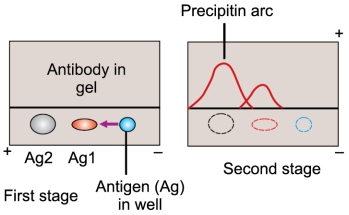


Fig. 13.6 Laurell's two-dimensional electrophoresis

B. AGGLUTINATION REACTION

When a **particulate antigen** is mixed with its antibody in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated. Agglutination is more sensitive than precipitation for the detection of antibodies. The same principles govern agglutination and precipitation. Agglutination occurs optimally when antigens and antibodies react in equivalent proportions. The zone phenomenon may be seen when either an antibody or an antigen is in excess. '**Incomplete**' or '**monovalent**' antibodies do not cause agglutination though they combine with the antigen. They may act as '**blocking**' antibodies, inhibiting agglutination by the complete antibody added subsequently.

Agglutination tests may be performed as slide agglutination or tube agglutination.

1. Slide Agglutination

Procedure

i) When a drop of the appropriate antiserum is added to a smooth, uniform suspension of a particulate antigen in a drop of saline on a slide or tile, agglutination takes place.

ii) A positive result is indicated by the clumping together of the particles and the clearing of the drop.

iii) The reaction is facilitated by mixing the antigen and the antiserum with a loop or by gently rocking the slide. Depending on the titre of the serum, agglutination may occur instantly or within seconds.

iv) Clumping occurring after a minute may be due to drying of the fluid and should be disregarded.

v) It is essential to have on the same slide, a control consisting of the antigen suspension in saline, without the antiserum, to ensure that the antigen is not autoagglutinable. Agglutination is usually visible to the unaided eye but may sometimes require confirmation under the microscope.

Uses of slide agglutination

• It is a routine procedure for the identification of many bacterial isolates from clinical specimens.
• It is also the method used for blood grouping and cross-matching.

2. Tube Agglutination

Agglutination test is performed in tubes when serial dilutions of the serum needs to be done as in febrile agglutination tests to measure antibodies. A fixed volume of a particulate antigen suspension is added to an equal volume of serial dilutions of an antiserum in test tubes; the agglutination titre of the serum can be estimated.

Uses of tube agglutination

• It is a routine procedure for the identification of many bacterial isolates from clinical specimens.
• It is also the method used for blood grouping and cross-matching.
• It is routinely used for the serological diagnosis of typhoid, brucellosis and typhus fever. In the Widal test used in typhoid, two types of antigens are used. The 'H' or flagellar antigen on combining with its antibody forms large, loose, fluffy clumps resembling wisps of cotton wool. The 'O' or somatic antigen forms tight, compact deposits resembling chalk powder. Agglutinated bacilli spread out in a disc-like pattern at the bottom of the tubes.

Limitations

- The agglutination test for brucellosis may be complicated by the **prozone phenomenon.**
- The presence of incomplete or '**blocking**' **antibodies** may be detected by the antiglobulin (Coombs) test, resulting in false negative results.
- Cross-reacting or **heterophile antigens**, which share similar antigenic epitopes between unrelated microorganisms may result in false positive results.

Some important agglutination tests for detecting antibodies to heterophile antigens are as follows:

- **Weil–Felix reaction** for serodiagnosis of typhus fevers is a heterophile agglutination test, which is based on the sharing of a common antigen between rickettsiae and some strains of *Proteus* bacilli.

- The ***Streptococcus* MG agglutination test** is used for the diagnosis of primary atypical pneumonia caused by *Mycoplasma pneumoniae*.
- **Paul–Bunnell test** and **cold agglutination test** are examples of agglutination tests using red cells as antigens. The former is based on the presence of sheep cell agglutinins in the sera of infectious mononucleosis patients, which are adsorbed by ox red cells but not by guinea pig kidney extract. The cold agglutination test is positive in mycoplasmal (primary atypical) pneumonia. The patient's serum agglutinates human O group erythrocytes at 4°C, the agglutination being reversible at 37°C.

Antiglobulin (Coombs) test

This test detects anti-Rh antibodies that do not agglutinate Rh-positive erythrocytes in saline.

Principle When sera containing incomplete anti-Rh antibodies are mixed with Rh-positive red cells, the incomplete immunoglobulin coats the surface of the erythrocytes but they are not agglutinated. When such erythrocytes coated with the anti-red cell antibody (gamma globulin) are washed free of all unattached protein and treated with a rabbit antiserum against human gamma globulin (anti-red cell gamma globulin or Coombs serum), the cells agglutinate (Fig. 13.7).

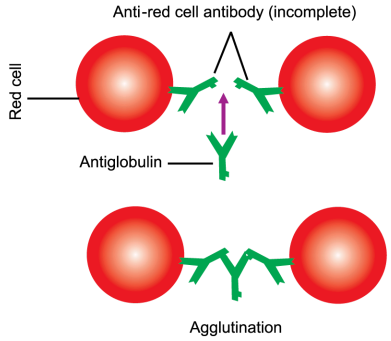


Fig. 13.7 Coombs test

Two types of Coombs test are available:

- ***Direct Coombs test*** The sensitisation of the erythrocytes with incomplete antibodies takes place in vivo, as in **hemolytic disease of the newborn due to Rh incompatibility**. When the red cells of erythroblastotic infants are washed free of unattached protein and then mixed with a drop of Coombs serum, agglutination results. The direct Coombs test is often negative in hemolytic disease due to ABO incompatibility.
- ***Indirect Coombs test*** Sensitisation of red cells with the gamma globulin is performed in vitro.

Uses of Coombs test

Originally employed for the detection of anti-Rh antibodies, the Coombs test is useful for demonstrating any type of incomplete or non-agglutinating antibody, as, for example, in brucellosis.

Passive agglutination test

By attaching **soluble antigens to the surface of carrier particles,** it is possible to convert precipitation tests into agglutination tests, which are more convenient and more sensitive for the detection of antibodies. Such tests are known as passive agglutination tests.

The commonly used carrier particles are red cells, latex particles or bentonite. Human or sheep erythrocytes adsorb on to a variety of antigens. Polysaccharide antigens may be adsorbed by simply mixing them with the cells. For adsorption of protein antigens, tanned red cells are used. Some types of passive agglutination tests are as follows:

- ***Hemagglutination*** A special type of passive hemagglutination test is the **Rose–Waaler test**. In rheumatoid arthritis, an autoantibody (RA factor) appears in the serum, which acts as an antibody to gamma globulin. The RA factor is able to agglutinate red cells coated with globulins. The antigen used for the test is a suspension of sheep erythrocytes sensitised with a sub-agglutinating dose of rabbit anti-sheep erythrocyte antibody (amboceptor).

- ***Latex agglutination test*** Polystyrene latex, which can be manufactured as uniform spherical particles, 0.8–1.0 μm in diameter, can adsorb several types of antigens. Latex agglutination tests (latex fixation tests) are widely employed in the clinical laboratory for the detection of anti-streptolysin O (ASO), C-reactive protein (CRP), RA factor, human chorionic gonadotropin (hCG) and many other antigens.
- ***Reversed passive agglutination*** Passive agglutination tests are very sensitive and yield high titres but may give false positive results. When, instead of the antigen, the antibody is adsorbed on to carrier particles in tests for the estimation of antigens, the technique is known as reversed passive agglutination. This method is used to diagnose bacterial antigens like *Legionella*, *Streptococcus pyogenes* and *N. gonorrhoeae* in clinical samples.
- ***Co-agglutination test*** It is based on the agglutination of *Staphylococcus aureus* bearing a specific antibody-sensitised protein A agglutinating with the soluble bacterial (e.g., *Legionella*) antigen in the clinical specimen.

C. COMPLEMENT FIXATION TEST (CFT)

Complement takes part in many immunological reactions and is absorbed during the combination of antigens with their antibodies. In the presence of the appropriate antibodies, the complement lyses erythrocytes, kills and, in some cases, lyses bacteria, immobilises motile organisms, promotes phagocytosis and immune adherence and contributes to tissue damage in certain types of hypersensitivity.

Principle The ability of antigen–antibody complexes to 'fix' complement is made use of in the CFT. This test is capable of detecting as little as 0.04 mg of antibody nitrogen and 0.1 mg of antigen. CFT is a complex procedure consisting of two steps and five reagents—antigen, antibody, complement, sheep erythrocytes and amboceptor (rabbit antibody to sheep red cells) (Fig. 13.8).

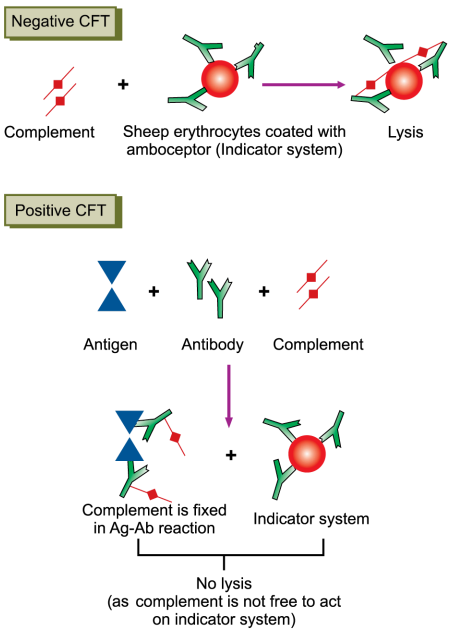


Fig. 13.8 Complement fixation test

Procedure

1. The antigen may be soluble or particulate. The antiserum is heated at 56°C (inactivated) for half an hour before the test to destroy any complement activity the serum may have and to remove some non-specific inhibitors of complement present in some sera (anti-complementary activity).
2. The source of the complement is guinea pig serum. It can be preserved either in the lyophilised or frozen state or with special preservatives, as in the Richardson's method.

Standardisation The guinea pig serum should be titrated for complement activity.

> **Minimum hemolytic dose** (MHD) or one unit of complement is defined as the highest dilution of guinea pig serum that lyses one unit volume of washed sheep erythrocytes in the presence of excess hemolysin (amboceptor) within a fixed time (usually 30 or 60 minutes) at a fixed temperature (37°C).

The amboceptor should be titrated for hemolytic activity. One MHD of amboceptor is defined as the least amount (or highest dilution) of the inactivated amboceptor which lyses one unit volume of washed sheep erythrocytes in the presence of excess complement within a fixed time (usually 30 or 60 minutes) at a fixed temperature (37°C). The diluent used for the titrations and for CFT is physiological saline with added calcium and

magnesium ions.

Appropriate controls should be used, including the following:

- **Antigen and serum controls** to ensure that they are not anti-complementary
- **Complement control** to ensure that the desired amount of complement is added
- **Cell control** to observe that sensitised erythrocytes do not undergo lysis in the absence of complement

Types of CFTs

1. ***Indirect complement fixation test*** Certain avian (e.g., duck, turkey and parrot) and mammalian (e.g., horse and cat) sera do not fix guinea pig complement. When such sera are to be tested, indirect complement fixation test may be employed. Here, the test is set up in duplicate and after the first step, the standard antiserum known to fix the complement is added to one set. If the test serum contains the antibody, the antigen will be used up in the first step and, therefore, the standard antiserum added subsequently cannot fix the complement. Therefore, in the indirect test, hemolysis indicates a positive result.
2. ***Conglutinating complement absorption test*** For systems which do not fix guinea pig complement, an alternative method is the conglutinating complement absorption test. This uses horse complement, which is non-hemolytic. The indicator system is sensitised sheep erythrocytes mixed with bovine serum. Bovine serum contains a beta globulin component called **conglutinin**, which acts as an antibody to the complement. Therefore, conglutinin causes agglutination of sensitised sheep erythrocytes (conglutination) if they have combined with the complement. If the horse complement had been used up by the antigen–antibody interaction in the first step, agglutination of sensitised cells will not occur.
3. ***Other complement-dependent serological tests*** When some bacteria (e.g., *Vibrio cholerae* and *Treponema pallidum*) react with the specific antibody in the presence of complement and particulate

materials such as erythrocytes or platelets, the bacteria are aggregated and adhere to the cells. This is known as **immune adherence**. The **immobilisation test** is another complement-dependent reaction. In the *Treponema pallidum* immobilisation test, the test serum is mixed with a live motile suspension of *T. pallidum* in the presence of the complement. On incubation, the specific antibody inhibits the motility of treponemes.

D. NEUTRALISATION TESTS

Neutralisation tests are based on the action of antibodies to neutralise an end product or the outcome of the action of an antigen.

1. ***Virus neutralisation tests*** Neutralisation of viruses by their antibodies can be demonstrated in various systems. It can be demonstrated by the plaque inhibition test, in which specific antiviral serum inhibits plaque formation by the virus.
2. ***Toxin neutralisation*** Bacterial exotoxins are good antigens and induce the formation of neutralising antibodies (antitoxins) which are important clinically in protection against diphtheria and tetanus. Toxin neutralisation can be tested in vivo.

Neutralisation tests in animals consist of injecting toxin–antitoxin mixtures and estimating the least amount of antitoxin, which prevents death or disease in the animals.

- The **Schick test** is based on the ability of a circulating antitoxin to neutralise the diphtheria toxin given intradermally, and indicates immunity or susceptibility to the disease. Toxin neutralisation in vitro depends on the inhibition of some demonstrable toxic effect.
- **Anti-streptolysin O (ASO) test** demonstrates that the antitoxin present in patient sera neutralises the hemolytic activity of the streptococcal O hemolysin (O, an immunogenic, oxygen-labile hemolytic toxin). When the body is infected with streptococci, it produces antibodies against the various antigens that the streptococci produce. ASO is one such antibody. Raised or rising levels indicate past or present infection.

- **Nagler's reaction** is an in vitro test for the identification of alpha toxin of *Clostridium perfringens* in clinical specimens. Action of the toxin lecithinase is neutralised by the antitoxin.

E. RADIOIMMUNOASSAY (RIA)

Some definitions pertaining to RIA are as follows:

Binder–ligand assay Besides fluorescent dyes, many other distinctive 'labels' such as radioisotopes and enzymes can also be conjugated with antigens and antibodies. The term binder–ligand assay has been used for these assays (e.g., RIA and enzyme immunoassays).

Analyte/ligand The substance (antigen) whose concentration is to be determined is called the analyte or ligand.

Binder The binding protein (ordinarily, the antibody) that binds to the ligand is called the binder.

RIA is a competitive binding assay in which fixed amounts of antibody and radiolabelled antigen react in the presence of unlabelled antigen. The labelled and unlabelled antigens compete for the limited binding sites on the antibody. This competition is determined by the level of the unlabelled (test) antigen present in the patient's serum samples. After the reaction, the antigen is separated into '**free**' and **'bound' fractions** and their radioactive counts are measured. The concentration of the test antigen can be calculated from the ratio of the bound and total antigen labels using a standard dose–response curve (Fig. 13.9).

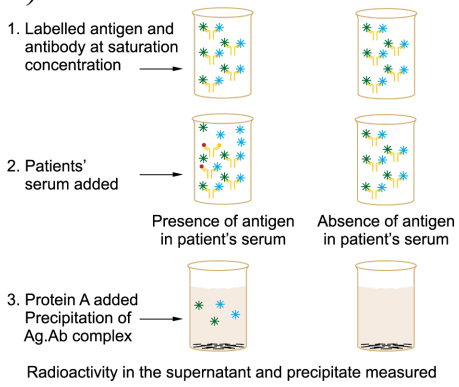

1. Labelled antigen and antibody at saturation concentration
2. Patients' serum added
Presence of antigen in patient's serum
Absence of antigen in patient's serum
3. Protein A added Precipitation of Ag.Ab complex
Radioactivity in the supernatant and precipitate measured

Fig. 13.9 Radioimmunoassay procedure

For any reacting system, the **standard dose–response** or **calibrating curve** has to be prepared first. This is done by running the reaction with

fixed amounts of antibody and labelled antigen, and varying known amounts of unlabelled antigen. The ratios of bound antigen to total antigen **(B:T ratio)** plotted against the analyte concentrations give the standard calibration curve
(Fig. 13.10). The concentration of antigen in the test sample is computed from the B:T ratio of the test by interpolation from the calibration curve. RIA and its modifications have applications in various areas of biology and medicine, including the quantitation of hormones, drugs, tumour markers, IgE and viral antigens. However, the hazards of radioisotope use have limited its applications.

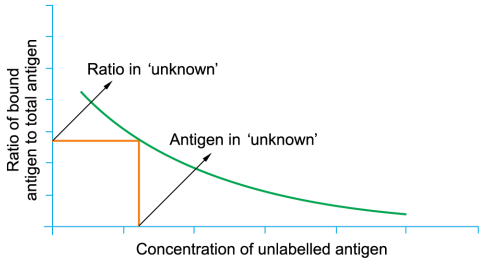


Fig. 13.10 Radioimmunoassay standard curve

F. ENZYME IMMUNOASSAY (EIA)

Enzyme-labelled antigens and antibodies were developed as serological reagents for the assay of antibodies and antigens. Their versatility, sensitivity, simplicity, economy and absence of radiation hazard have made EIA the most widely used procedure in clinical serology. The availability of test kits and facility for automation has added to their popularity.

The term enzyme immunoassay includes all assays based on the measurement of enzyme-labelled antigen, hapten or antibody. EIAs are of two basic types:

- **Homogeneous EIA** does not require the bound and free fractions to be separated; the test can thus be completed in one step, with all reagents added simultaneously. This type of EIA can be used only for the assay of haptens such as drugs and not for microbial antigens and antibodies. An example of homogeneous EIA is **enzyme-multiplied immunoassay technique (EMIT)**, which is a simple assay method for small-

molecule drugs such as opiates, cocaine, barbiturates or amphetamine in serum.

- **Heterogeneous** EIA requires the separation of the free and bound fractions either by centrifugation or by adsorption on solid surfaces and washing. It is, therefore, a multistep procedure, with reagents added sequentially. The main type of heterogeneous EIA is **ELISA**.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is so named because the technique involves the use of an **immunosorbent**—an absorbing material specific for one of the components of the reaction: the antigen or the antibody. This material may be particulate, for example, cellulose or agarose, or a solid phase such as polystyrene, polyvinyl or polycarbonate tubes or microwells, membranes or discs of polyacrylamide, paper or plastic. ELISA is usually done using 96-well microtitre plates suitable for automation. The principle of the test can be illustrated by outlining its application for the detection of rotavirus antigen in feces.

Types of ELISA

1. ***Indirect ELISA*** The **detection of antibody** by ELISA can be illustrated by the anti-HIV antibody test. Purified inactivated HIV antigen is adsorbed onto microassay plate wells. Test serum diluted in a buffer is added to the well and incubated at 37°C for 30 minutes. The well is then thoroughly washed. If the serum contains anti-HIV antibody, it will form a stable complex with the HIV antigen on the plate. A goat anti-human immunoglobulin antibody conjugated with horseradish peroxidase (HRP) enzyme is added and incubated for 30 minutes. After thorough washing, the substrate *o*-phenylenediamine dihydrochloride is added. After 30 minutes, the colour that develops is read using a microassay plate reader. Positive and negative controls should be used with test sera (Fig. 13.11).
2. ***Competitive ELISA*** Similar to RIA, both the unknown antigen (sample) and the known antigen (standard) compete with each other for a fixed amount of antibody. Competitive ELISA yields an inverse

curve, where higher values of antigen in the samples/standards yield a lower amount of colour change. It is normally used for hapten detection (Fig. 13.11).

3. ***Sandwich ELISA*** It is used for antigen detection in the patient sample. The antigen is sandwiched between two layers of antibodies (i.e., capture and detection antibodies).

Procedure for sandwich ELISA

The steps in the sandwich ELISA procedure of rotavirus detection, for example, are as follows:

i) The wells of a microtitre plate are coated with goat antirotavirus antibody. After thorough washing, the fecal samples to be tested are added and incubated overnight at 4°C or for two hours at 37°C. Suitable positive and negative controls are also set up.

ii) Guinea pig antirotavirus antiserum, labelled with alkaline phosphatase, is added and incubated at 37°C for one hour.

iii) After washing, a suitable substrate (*para*-nitrophenylphosphate) is added and held at room temperature until the positive controls show the development of a yellow colour. The phosphatase enzyme splits the substrate to yield a yellow compound.

iv) If the test sample contains rotavirus, it is fixed to the antibody coating the wells. When the enzyme-labelled antibody is added subsequently, it is in turn, fixed. The presence of residual enzyme activity, indicated by the development of yellow colour, therefore, denotes a positive test.

v) If the sample is negative, there is no significant colour change. An ELISA reader provides quantitative colour recordings which are directly proportional to the quantity of the analyte present in the test sample (Fig. 13.11).

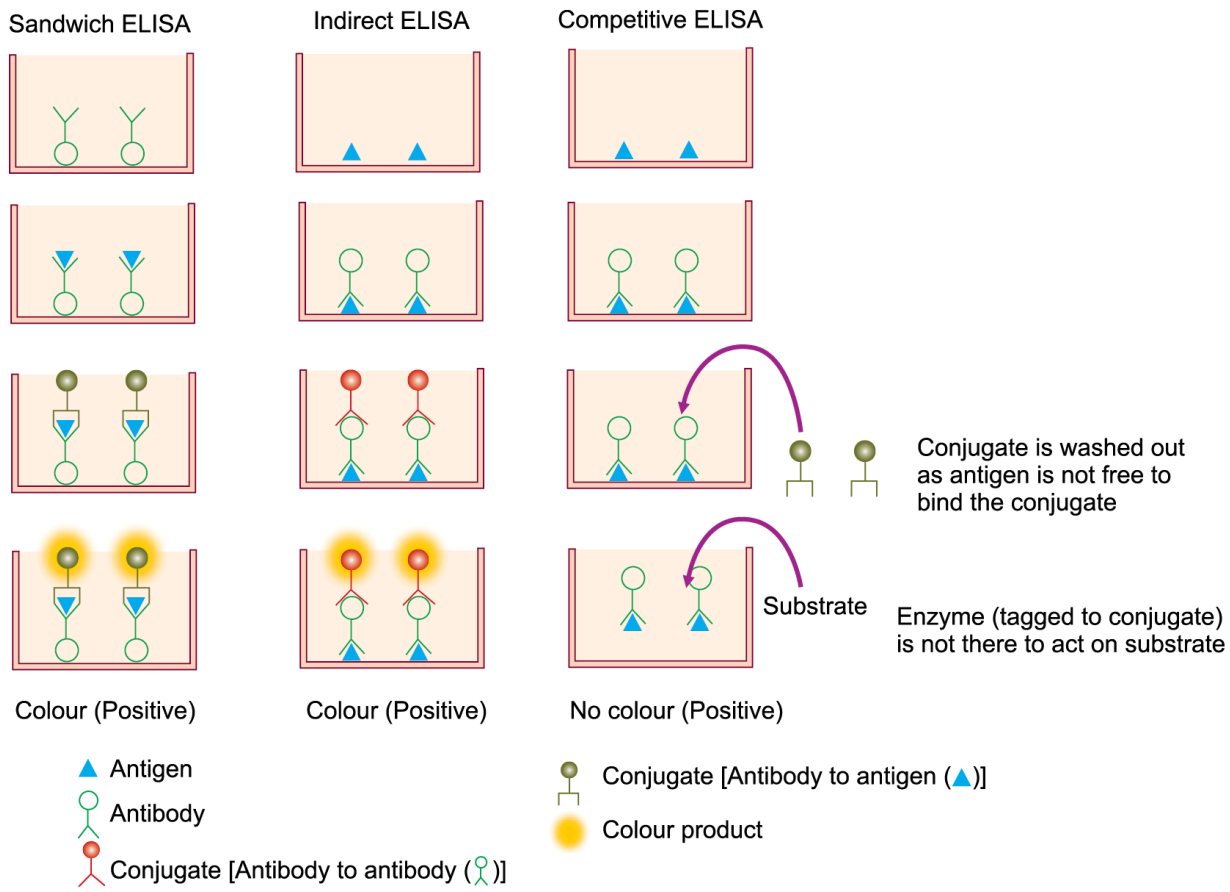


Fig. 13.11 Enzyme-linked immunosorbent assay (ELISA)

4. ***Capture ELISA*** This test and immunometric tests are even more specific. Several variations of the ELISA technique have been developed to provide simple diagnostic tests, including the card and dipstick methods suitable for clinical laboratory and bedside applications. For example, monoclonal anti-human IgM antibodies are coated on wells which bind to IgM from the patient's serum. The complex binds to specific antigens against which the antibody is being detected. Then, the antigen-specific mouse monoclonal antibody (MAb), which is conjugated with HRP, is added and the complex is detected based on the enzyme-substrate reaction by a spectrophotometer.
5. ***Cylinder or cassette ELISA*** A simple modification of ELISA which has found wide application for testing one or a few samples of sera at a time is the cylinder or cassette ELISA. Here, each specimen is tested in a separate disposable cassette. The test is rapid (10–15 minutes) and there is no need for microplate washers or readers. The

result is read visually. In-built positive and negative controls are usually provided for validation of the test procedure.

Dot blot assay This is an example of cassette ELISA used for the detection of HIV type 1 and 2 antibodies. Specific type 1 and 2 antigens are immobilised at separate fixed sites on the nitrocellulose membrane in the cassette.

Procedure Test serum is added on the nitrocellulose membrane and allowed to filter into the absorbent material placed below it in the cassette base. Antibody, if present in the serum, will bind to the appropriate antigen. After washing to remove the unbound antibody, enzyme-labelled anti-human immunoglobulin antibody is added. After additional washing to remove the unbound conjugate, a substrate which yields a coloured product is added.

Result A positive result is indicated by a coloured spot developing at the site of the antigen against which the antibody is present in the serum. Human immunoglobulin immobilised at a spot on the membrane acts as a control for the test procedure, as shown by the development of colour at the site.

6. ***Enzyme-linked immunospot assay (ELISpot)*** This is a highly sensitive method to detect the secreted protein, e.g., cytokines from cytokine-secreting cells or Ig from immunoglobulin-secreting cells. The secreted molecules are detected using a procedure similar to ELISA.
7. ***IgG avidity ELISA*** This test discriminates acute from chronic infection based on the principle of immunoenzyme assay in which a destabilising agent such as urea is used to dissociate the binding between the specific IgG and the antigen. In recent infection, the avidity of the antibody to bind is weaker and so it dissociates from the antigen–antibody complex; in chronic infection, the binding is stronger, hence there is no dissociation from the antigen. The result is expressed as **avidity index**. The test is used to detect recent infection of rubella, toxoplasma or cytomegalovirus in pregnant women.

Uses of ELISA

ELISA plays a major role in the diagnosis of innumerable diseases. Some examples are given below:
- Infectious diseases like hepatitis, EBV, cytomegalovirus IgM/IgG, dengue IgG, influenza, TORCH panel, hepatitis B, C, etc.
- Rotavirus detection in fecal specimens and enterotoxin of E.coli in feces
- Syphilis IgG/IgM, H. pylori IgG and antigen detection
- Food toxins like chloramphenicol, streptomycin, penicillin, aflatoxins, etc.
- Food adulterants including E. coli, Campylobacter and Salmonella antigens
- Human allergen-specific IgE and IgA ELISA

G. Chemiluminescence immunoassay (CLIA)

Chemiluminescence refers to a chemical reaction emitting energy in the form of light. Just as radioactive conjugates are employed in RIA, fluorescent conjugates in IFA and enzymes in ELISA, chemiluminescent compounds (such as luminol or acridinium esters) are used in CLIA as the label to provide the signal during the antigen–antibody reaction. The signal (light) can be amplified, measured and the concentration of the analyte calculated. The method has been fully automated and is being increasingly used in laboratories where the volume of work is large.

H. IMMUNOELECTROBLOT / WESTERN BLOT TECHNIQUES

Immunoelectroblot or **western blot** techniques combine the sensitivity of enzyme immunoassay with much greater specificity. The technique is a combination of three separate procedures:

- **Separation of ligand–antigen components** by polyacrylamide gel electrophoresis
- **Blotting of the electrophoresed ligand fraction** on nitrocellulose membrane strips
- **Enzyme immunoassay** (or radioimmunoassay) to:
    - Detect antibody in test sera against the various ligand fraction bands
    - Probe with known antisera against specific antigen bands

The western blot test, considered to be the definitive/confirmatory test for

the serodiagnosis of HIV infection, is an example of the immunoelectroblot technique.

I. IMMUNOCHROMATOGRAPHIC ASSAY / LATERAL FLOW ASSAY

This is a one-step, qualitative immunochromatographic technique that has found wide application in serodiagnosis due to its rapidity, simplicity, economy and reliability. A description of its use for HBsAg detection illustrates the method (Fig. 13.12).

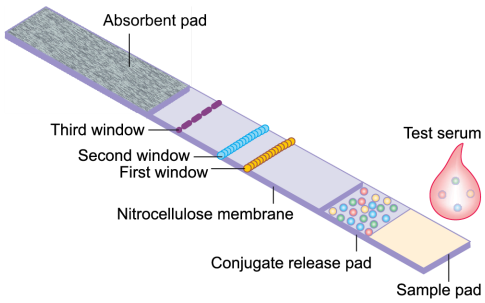


Fig. 13.12 Lateral flow immunoassay

Procedure The test system is a small cassette containing a membrane impregnated with anti-HBsAg antibody – colloidal gold dye conjugate. The membrane is exposed at three windows on the cassette. The test serum is dropped into the first window. As the serum travels upstream by capillary action, a coloured band appears at the second window (test site) if the serum contains HBsAg, due to the formation of an HBsAg–antibody conjugate complex. This is the positive reaction. The absence of a coloured band at the test site indicates a negative reaction. Simultaneously, a coloured band should appear in every case at the third window, which forms an in-built control and in the absence of which, the test is invalid. The test is claimed to be nearly as sensitive and specific as EIA tests. **Several rapid ICT tests for viral, parasitic and bacterial infections are used in diagnosis.**

J. IMMUNOELECTRON MICROSCOPIC ASSAYS

1. ***Immunoelectron microscopy*** When viral particles mixed with specific antisera are observed under the electron microscope, they are seen to be clumped. This finds application in the study of some

viruses such as the hepatitis A virus and the several viruses that cause diarrhea.

2. ***Immunoferritin test*** Ferritin (an electron-dense substance from horse spleen) can be conjugated with antibody, and such labelled antibodies reacting with antigens can be viewed under the electron microscope.
3. **Immunoenzyme test** Some stable enzymes, such as peroxidase, can be conjugated with antibodies. Tissue sections carrying the corresponding antigens are treated with peroxidase-labelled antisera. The peroxidase bound to the antigen can be visualised under the electron microscope by immunohistochemical methods. Some other enzymes, such as glucose oxidase, phosphatases and tyrosinase, may also be included in immunoenzyme tests.

K. IMMUNOFLUORESCENCE ASSAY

Fluorescence is the property of absorbing light rays of one particular wavelength and emitting rays with a different wavelength. Fluorescent dyes show up brightly under ultraviolet light as they convert ultraviolet light into visible light. Fluorescent dyes can be conjugated to antibodies and then be used to locate and identify antigens in tissues. This 'fluorescent antibody' or immunofluorescence technique has several diagnostic and research applications
(Fig. 13.13).

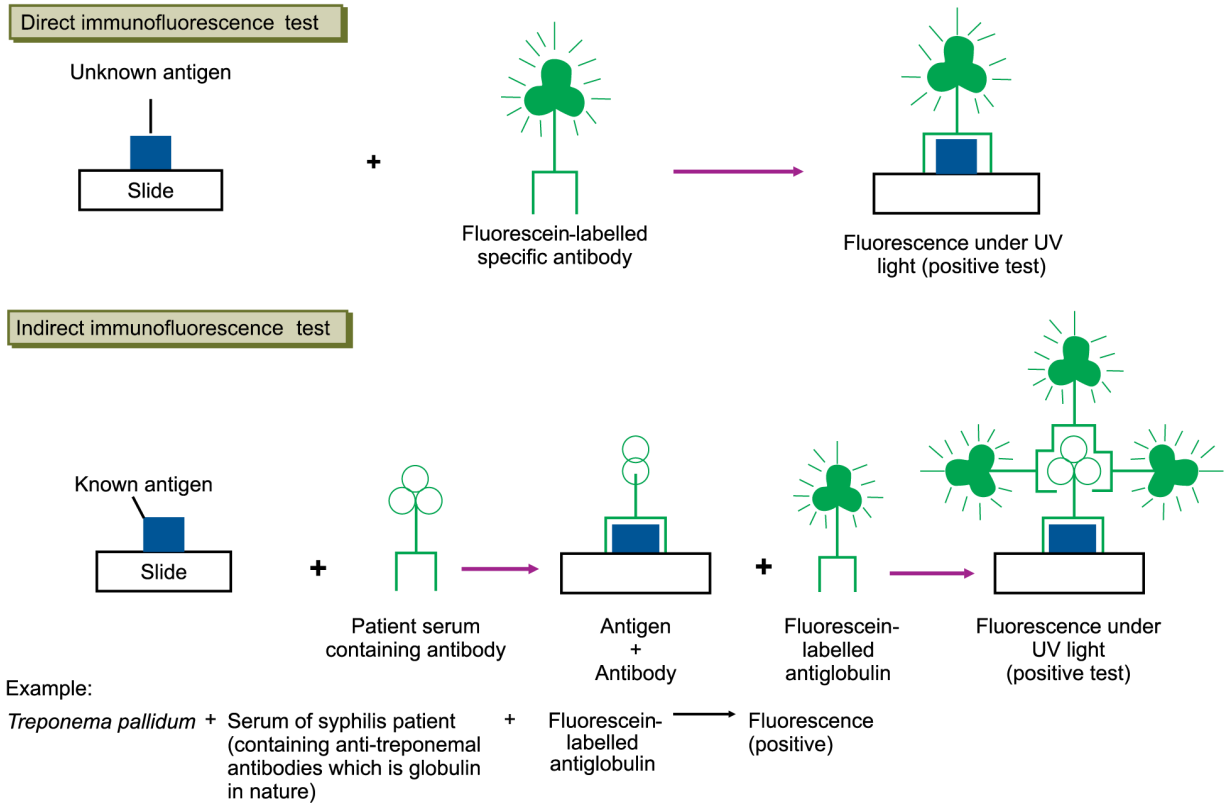


Fig. 13.13 Direct and indirect immunofluorescence tests

1. ***Direct immunofluorescence test*** This can be used for the identification of bacteria, viruses or other antigens, using the specific antiserum labelled with a fluorescent dye. For example, direct immunofluorescence is routinely used as a sensitive method for diagnosing rabies, by detection of the rabies virus antigens in brain smears. A disadvantage of this method is that separate fluorescent conjugates have to be prepared against each antigen to be tested.
2. ***Indirect immunofluorescence test*** This test overcomes the difficulty mentioned above by using an antiglobulin fluorescent conjugate. This antiglobulin conjugate tags to the antibody fixed to the antigen. An example is the fluorescent treponemal antibody (FTA) test for the diagnosis of syphilis.

Fluorescent dyes may also be conjugated with the complement. Labelled complement is a versatile tool and can be employed for the detection of antigen or antibody. Antigens also take fluorescent labelling but not as well as antibodies do. For the detection of antibodies by immunofluorescence, the **sandwich** technique can be used. The antibody is first allowed to react

with unlabelled antigen, which is then treated with fluorescent-labelled antibody. A sandwich is thus formed, the antigen being in the middle and the labelled and unlabelled antibodies on its either side.

Immunohistochemical Techniques

By combining the specificity of serology with the localising capacity of histology, immunofluorescence helps in the visualisation of antigen–antibody reactions in situ. Such techniques are thus called immunohistochemical techniques. The fluorescent dyes commonly used are **fluorescein isothiocyanate** and **rhodamine**, which exhibit blue-green and orange-red fluorescence, respectively.

Flow Cytometry

This is a fluorescence technique used to identify and enumerate cells bearing a particular antigen(s) or the surface markers by suspending them in a stream of fluid and passing them through an electronic detection apparatus. It allows simultaneous, multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Different populations of molecules, cells or particles can be differentiated by size and shape using forward and right-angled light scatter. These cells, particles or molecules can be labelled with different fluorescent labels or with dye-labelled monoclonal antibodies. In this way, it is possible to measure the amounts or isolated individual cells or populations of particular cells in a mixed population.

The cells are made to flow in a single cell stream in a flow cell by hydrodynamic focusing. The sample stream containing the cells is focussed by a surrounding layer of sheath fluid. An Argon laser (at 488 nm) is focussed on the cells. Various sensors pick up the laser light reflected by the cells.

The parameters measured by the flow cytometer are forward scatter (FS), a measure of cellular size, side scatter (SS), a measure of the granularity of the cell and fluorescence sensors (FL1–FL8), which measure light emitted by various dyes bound to the cells. The emitted light is directed into various sensors by a combination of filters (Fig. 13.14).

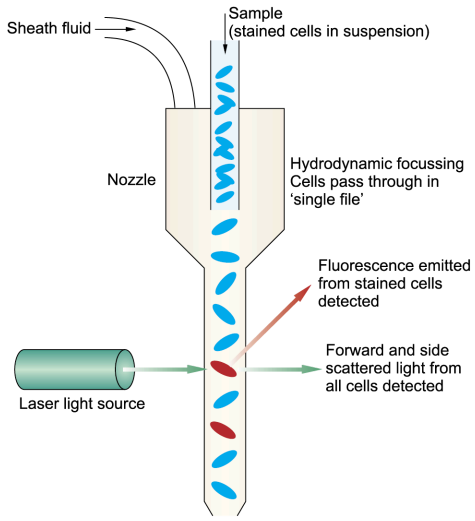


Fig. 13.14 Schematic diagram of a flow cytometer

Applications of flow cytometry

- Measurement of multiple parameters using flow cytometry, including:
  - Size
  - Granularity
  - DNA or RNA content
  - Cellular antigens
  - Receptor levels of cells
- Wide use in research and diagnostics:
  - To count blood cells including differential leucocyte count (DLC)
  - To isolate T cell subsets (CD4 and CD8 counts in HIV patients)
  - For diagnosis, treatment and prognosis in cancer (especially leukemias)
  - To study the cell cycle and apoptosis

Key points

- Antigen–antibody reactions make it possible to detect, identify and quantify (measure the concentration) of antigens and antibodies. Since antibodies are present in serum, their study is known as serology.
- Antibodies may be demonstrated by various types of reactions:
  - Agglutination reactions: In these reactions, specific antibodies (agglutinins) formed in response to the occurrence of particulate antigens in host tissues combine with a homologous antiserum. Agglutination reactions are used for the diagnosis of infections due to salmonellae (Widal test), brucellae (brucella agglutination test), and rickettsiae (Weil–Felix test) and other toxic products elaborated by microorganisms.
  - Precipitation reactions: In these reactions, the antigen is in a soluble form and, on combination with the antibody, forms sediments or remains suspended in the form

of floccules. Examples of diagnostic precipitation tests include the Kahn and VDRL tests.

- Complement fixation reaction: In this type of reaction, an antigen combines with its (specific) antibody in the presence of complement, and the antigen–antibody complex adsorbs the complement. This reaction is not visible and requires the use of an indicator (sheep erythrocytes and the specific anti-erythrocyte antibody). Examples include the Wassermann test for syphilis and some tests for the detection of viruses.
- Neutralisation tests: In these tests, the effect of the antigen, toxin or virus is neutralised on mixing with its antibody. Examples include the Schick test for diphtheria toxin, antistreptolysin O (ASO) test for streptococcal O infection, Nagler's reaction for the identification of alpha toxin of *Clostridium perfringens* and the *Treponema pallidum* immobilisation (TPI) test for *T. pallidum* in clinical specimens.
- In certain viral diseases, the antibody produced prevents the agglutination of certain red blood cells by the specific virus; this hemagglutination inhibition test can be used for the diagnosis of influenza.

- Tests based on the primary interaction between the antibody and antigen are very sensitive and specific. In radioimmunoassay, a radioisotope is used to indicate the presence of an antigen–antibody reaction.
- Enzyme immunoassay (EIA) is a detection system that involves the use of an enzyme-labelled antibody. ELISA is the most popular technique for the diagnosis of hepatitis, HIV, rotavirus and many others.
- Chemiluminescence refers to a chemical reaction emitting energy in the form of light.
- Immunofluorescence technique involves fluorochrome-labelled antibodies for the diagnosis of syphilis, treponemal infection, etc., in clinical samples and also visualisation of antigen–antibody reactions in situ by the immunohistochemical technique.
- Flow cytometry allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of cells. Different populations of cells or particles can be differentiated by size and shape using forward and light scatter, where cells are made to flow in a single cell stream. It is widely used for CD4 and CD8 counts in HIV patients, diagnosis and prognosis in cancer (especially leukemias), study of cell cycle and apoptosis, etc.

Questions

Essays

1. Enumerate the types of antigen–antibody reactions and describe the principle and applications of the precipitation reactions.
2. Describe in detail the agglutination test and its use in laboratory diagnosis.

Short Answers

1. Precipitation reactions

2. Agglutination test
3. Complement fixation test
4. Neutralisation test
5. Immunofluorescence test
6. Principle and applications of ELISA in clinical microbiology
7. Principle of immunoelectrophoresis
8. Principle of radioimmunoassay and its advantages and disadvantages over ELISA
9. Rocket immunoelectrophoresis
10. Principle of Coombs test (antiglobulin test)
11. Ouchterlony double immunodiffusion technique
12. Two-dimensional electrophoresis
13. Sandwich ELISA
14. Immunoelectroblot/Western blot technique
15. Flow cytometry

Part II: Immunology

Chapter 14

Hypersensitivity

Chapter navigation

- Type I (immediate anaphylactic hypersensitivity)
- Type II (cytolytic/cytotoxic hypersensitivity)
- Type III (immune complex-mediated hypersensitivity)
- Type IV (delayed type T cell-mediated hypersensitivity)

INTRODUCTION

Immune response is generally aimed at removing antigens using various effector molecules without damaging the host tissue. However, in some instances, the inflammatory response can have harmful effects like an exaggerated inflammatory response or tissue injury, sometimes even leading to death. This heightened response is known as **hypersensitivity.** This occurs during the second contact with the specific antigen **(allergen).**

The term **hypersensitivity** refers to the undesirable injurious consequences in the sensitised host following contact with specific antigens. The antigens are often innocuous substances such as serum proteins or pollen. **Hypersensitivity is concerned with what happens to the host as a result of an inappropriate immune reaction.**

The term **allergy** refers to all immune processes harmful to the host, such as hypersensitivity and autoimmunity.

The host should have had prior contact with the antigen (allergen) in order to induce hypersensitivity reactions. The initial contact sensitises the immune system, leading to priming of the appropriate B or T lymphocytes. This is known as the **sensitising** or **priming dose**. Subsequent contact (known as the **shocking dose)** with the allergen results in manifestations of hypersensitivity.

CLASSIFICATION OF HYPERSENSITIVITY REACTIONS

Classification Based on Time

Hypersensitivity reactions have been classified traditionally into **'immediate'** and **'delayed'**, based on the time required for a sensitised host to develop clinical reactions on re-exposure to the antigen. The main differences between these types of hypersensitivity reactions are shown in Table 14.1.

Table 14.1 Distinguishing features of immediate and delayed types of hypersensitivity

Immediate hypersensitivity	Delayed hypersensitivity
Appears and recedes rapidly	Appears slowly, lasts longer
Induced by antigens or haptens by any route	Induced by antigen or hapten introduced intradermally or with Freund's adjuvant or by skin contact
This is an antibody-mediated reaction involving circulating antibodies	The reaction is cell-mediated; circulating antibodies may be absent
Passive transfer is possible with transfer of serum to a recipient	This is possible with transfer of T cells or transfer factor and cannot be transferred with serum
Desensitisation is easy but short-lived	Desensitisation is difficult but long-lasting

Classification Based on Mechanism

Hypersensitivity reactions are classified into four types based on the different mechanisms of pathogenesis (described by **Coombs and Gell** in 1963). Types I, II and III are grouped under immediate hypersensitivity while type IV is considered as delayed hypersensitivity.

A. Immediate hypersensitivity

These types are B cell- or antibody-mediated and are as follows:

- ***Type I (immediate anaphylactic hypersensitivity)*** Antibodies ('cytotropic' IgE antibodies) are fixed on the surface of tissue cells (mast cells and basophils) in sensitised individuals. The antigen combines with the cell-fixed antibody, leading to the release of pharmacologically active substances (vasoactive amines), which produce the clinical reaction.
- ***Type II (cytolytic/cytotoxic hypersensitivity)*** The mechanism of this reaction is antibody-mediated cell damage. This type of reaction is initiated by IgG (or rarely, IgM) antibodies that react either with cell-surface or tissue antigens. Cell or tissue damage

occurs in the presence of complement or mononuclear cells. Type II reactions are intermediate, between hypersensitivity and autoimmunity. Combination with antibody may, in some instances, cause stimulation instead of damage. **In stimulatory hypersensitivity (referred to as Type V)**, antibodies recognise and bind to the cell-surface receptors. This is a modified form of Type II reaction.

- ***Type III (immune complex-mediated hypersensitivity)*** Here, the damage is caused by antigen–antibody complexes causing complement activation. The antibody is the high-affinity IgM and IgG that bind to the antigen. These complexes may precipitate in and around small blood vessels, causing damage to cells secondarily, or on membranes, interfering with their function. They may relate to tissue deposition (**Arthus phenomenon**), for example vasculitic (skin), serum sickness (systemic), nephritis (kidneys) and extrinsic allergic alveolitis (lungs).

B. Delayed hypersensitivity

- ***Type IV (delayed type T-cell mediated hypersensitivity)*** This is a cell-mediated response seen in tuberculin test and contact dermatitis. The antigen activates specifically sensitised CD4 and CD8 T cells, leading to the secretion of lymphokines and phagocyte accumulation, which in turn, manifest as delayed type of hypersensitivity (DTH).

The classification and some of the features of hypersensitivity reactions are shown in Table 14.2. The four types of immunopathogenic mechanisms described here are not mutually exclusive. Any given hypersensitive reaction may consist of components of more than one, or all, of these mechanisms.

The pathology and clinical features of such immunological diseases would also be influenced by the **humoral amplification system,** which collectively contributes to many of the non-immune body mechanisms such as inflammation, complement, coagulation, fibrinolytic and kininogenic

systems.

Table 14.2 Types of hypersensitivity reactions and their features

Type of reaction	Clinical syndrome	Time required for manifestation	Mediators
Type I: IgE type	1. Anaphylaxis	Minutes	IgE, histamine and other pharma cological agents
	2. Atopy Respiratory: asthma, eczema, hay fever GI: Allergy to shellfish Familial or genetic predisposition	Minutes	
Type II: Cytolytic and cytotoxic	Antibody-mediated damage — thrombocytopenia— agranulocytosis, hemolytic anemia, etc.	Variable: hours to days	IgG, IgM, complement
Type III: Immune complex	1. Arthus reaction	Variable: hours to days	IgG, IgM, complement, leucocytes
	2. Serum sickness	Days	
Type IV: Delayed hypersensitivity	1. Tuberculin	Hours to days	T cells, lymphokines, macrophages
	2. Contact dermatitis	Hours to days	

TYPE I REACTIONS (IMMEDIATE ANAPHYLACTIC HYPERSENSITIVITY)

These occur in two forms: the acute, potentially fatal, systemic form called **anaphylaxis** and the chronic or recurrent, non-fatal and typically localised form called **atopy**.

Anaphylaxis

This is the classical immediate hypersensitivity reaction which occurs within seconds of the antigen combining with IgE bound to mast cells, basophils and possibly, eosinophils (Fig. 14.1).

- There should have been prior exposure to the antigen with an interval of at least 2–3 weeks between the sensitising dose and

the shocking dose. Once sensitised, the individual remains so for long periods.

- The shocking dose is most effective when injected intravenously, less effective intraperitoneally or subcutaneously and least effective intradermally.

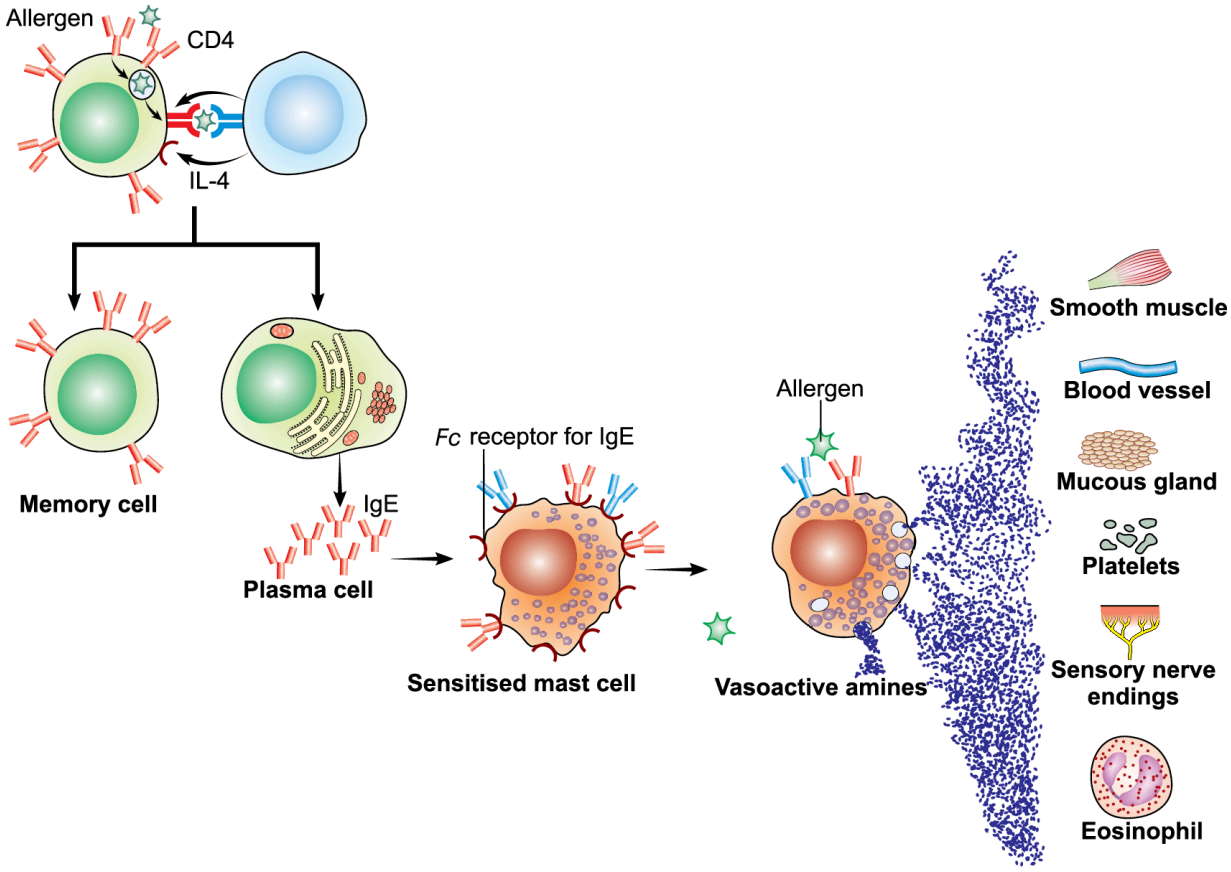


Fig. 14.1 Type I reaction: Immediate anaphylactic hypersensitivity reaction mediated by IgE

Mechanism of Type I Hypersensitivity

The immunological basis of hypersensitivity is the cytotropic IgE antibody. Free IgE antibody in circulation is not relevant in anaphylaxis. Anaphylaxis is caused by a cell-fixed antibody, even in the absence of detectable circulating antibodies. While in human beings, IgE is the cytophilic antibody, in guinea pigs and mice, the analogous cytophilic antibody is IgG_1.

During the first exposure to an allergen (antigen), plasma cells secrete IgE in response to activation by the allergen-specific T_h2 cells. This phase is

known as **sensitisation**. IgE molecules are bound to surface *Fc* epsilon receptors (*Fc*εRI), which are high-affinity immunoglobulin receptors expressed on the surface of tissue mast cells and blood basophils. FcεRI is a tetrameric membrane protein complex, which is present on the surface of mast cells and basophils. These receptors are analogous to the TCR receptors on the T cell surface. IgE molecules attach to these receptors by their *Fc* end. On subsequent effector phase, an exposure to the same allergen causes cross-linking of the cell-bound IgE, which leads to the bridging of the gap between adjacent antibody molecules. This cross-linking increases the permeability of the cells to calcium ions and leads to degranulation, releasing biologically active substances contained in the granules.

Pharmacological Mediators

The **primary mediators** are the pre-formed contents of mast cells and basophils.

1. ***Histamine*** This is the most important vasoactive amine in human anaphylaxis. Histamine is formed by the decarboxylation of histidine found in the granules of mast cells, basophils and platelets. Released into the skin, histamine stimulates sensory nerves, producing burning and itching sensations. It causes vasodilatation and hyperemia by an axon reflex (flare effect) and edema by increasing capillary permeability (wheal effect). Histamine induces smooth muscle contraction in diverse tissues and organs.
2. ***Serotonin (5-hydroxytryptamine)*** This is a base derived by decarboxylation of tryptophan. It is found in the intestinal mucosa, brain tissue and platelets.
   It causes smooth muscle contraction, increased capillary permeability and vasoconstriction.
3. ***Chemotactic factors*** The eosinophil chemotactic factors of anaphylaxis (ECF-A) are acidic tetrapeptides released from mast cell granules, which are strongly chemotactic for eosinophils. These probably contribute to the eosinophilia accompanying many hypersensitivity states. A high-molecular-weight chemotactic factor which attracts neutrophils (NCF) has been identified.

4. ***Enzymatic mediators*** Enzymes such as proteases and hydrolases are also released from mast cell granules.
5. ***Secondary mediators*** They are newly formed upon stimulation by mast cells, basophils and other leucocytes.
6. ***Prostaglandins and leukotrienes*** They are derived by two different pathways from arachidonic acid, which is formed from disrupted cell membranes of mast cells and other leucocytes. The lipoxygenase pathway leads to the formation of leukotrienes, while the cyclo-oxygenase pathway leads to the formation of prostaglandins and thromboxane. A substance originally demonstrated in the lungs, producing slow, sustained contraction of smooth muscles, and therefore termed slow-reacting substance of anaphylaxis (SRS-A), has since been identified as a family of leukotrienes (LTB4, C4, D4 and E4). Prostaglandin F2α and thromboxane A2 are powerful but transient bronchoconstrictors. Prostaglandins also affect secretion by mucous glands, platelet adhesion, permeability and dilatation of capillaries and the pain threshold.
7. ***Platelet-activating factor (PAF)*** PAF is a low-molecular-weight lipid released from basophils, which causes aggregation of platelets and release of their vasoactive amines.
8. ***Other mediators of anaphylaxis*** Several biologically active substances, such as the anaphylatoxins released by complement (C3a, C5a) activation, bradykinin and other kinins formed from plasma kininogens, mediate anaphylaxis.

Anaphylactoid reaction
Intravenous injection of peptone, trypsin and certain other substances provokes a clinical reaction resembling anaphylactic shock. This is termed an anaphylactoid reaction. The only difference is that anaphylactoid shock has no immunological basis and is a non-specific mechanism involving the activation of complement and the release of anaphylatoxins.

The organs affected in anaphylaxis vary with the species. Tissues or organs predominantly involved in the anaphylactic reaction are known as '**target tissues**' or '**shock organs**'. Other **changes seen in anaphylaxis are edema, decreased coagulability of blood, fall in blood pressure and temperature, leucopenia and thrombocytopenia**.

Signs and Symptoms

In human beings, fatal anaphylaxis is fortunately rare. Symptoms and signs of anaphylactic shock begin with itching of the scalp and tongue, flushing of the skin over the whole body and difficulty in breathing due to bronchial spasm. Nausea, vomiting, abdominal pain and diarrhea, sometimes with blood in the stool, may be present. Acute hypotension, loss of consciousness and death follow. Human anaphylaxis, once commonly associated with heterologous serum therapy, is now seen mostly following the injection of antibiotics or other drugs. Insect stings can also cause anaphylaxis in human beings.

Treatment

Prompt treatment with adrenaline can be life-saving (0.5 mL of a 1-in-1,000 solution, subcutaneously or intramuscularly).

Types

1. Cutaneous anaphylaxis

When a small, shocking dose of an antigen is administered intradermally to a sensitised host, a local **wheal-and-flare** response (local anaphylaxis) occurs. A wheal is a pale, central area of puffiness due to edema, which is surrounded by a flare caused by hyperemia and subsequent erythema. Skin test is useful in testing for Type I hypersensitivity and in identifying the allergen responsible in atopic diseases. In highly sensitised individuals, even the skin test may lead to serious and even fatal reactions. Hence a syringe loaded with adrenaline should always be kept ready (refer to *Clinical case*).

Clinical Case

A 45-year-old farmer was brought to the Emergency department with shortness of breath, pounding sensation in the head and tightness in the chest. He had a history of being stung by bees on two earlier occasions. The clinical diagnosis was of cutaneous anaphylaxis. Intramuscular adrenaline and intravenous antihistamines were administered. The patient recovered completely by the following day.

2. Passive cutaneous anaphylaxis (PCA)

This test, which was developed by Ovary (1952), is an extremely sensitive

in vivo method for the detection of antibodies. A small volume of the antibody is injected intradermally into a normal animal. If the antigen, along with a dye such as Evans blue, is injected intravenously 4–24 hours afterwards, there will be immediate blue colouration at the site of intradermal injection due to vasodilatation and increased capillary permeability (wheal-and-flare reaction). PCA can be used to detect the human IgG antibody, which is heterocytotropic (capable of fixing to cells of other species) but not IgE, which is homocytotropic.

3. Anaphylaxis in vitro

Isolated tissues, such as intestinal or uterine muscle strips from sensitised guinea pigs, held in a bath of Ringer's solution will contract vigorously on the addition of the specific antigen to the bath. This is known as the **Schultz–Dale phenomenon**. Tissues from normal animals can be passively sensitised by treatment with serum from sensitised animals.

Atopy

This refers to naturally occurring **familial hypersensitivities** of human beings, typified by hay fever and asthma. The antigens commonly involved in atopy are characteristically environmental inhalants (e.g., pollen and house dust) or ingestants (e.g., eggs and milk). Contact allergens to which the skin and conjunctiva may be exposed can also cause atopy. These atopens are generally not good antigens when injected parenterally but induce IgE antibodies, formerly termed 'reagin' antibodies. Atopic sensitisation is developed spontaneously following natural contact with atopens. It is difficult to induce atopy artificially.

Predisposition to atopy is **genetically determined**, probably linked to MHC genotypes. Atopy, therefore, runs in families. What is inherited is not sensitivity to a particular antigen or a particular atopic syndrome but the tendency to produce IgE antibodies in unusually large quantities.

All individuals are capable of forming IgE antibodies in small amounts but in atopics, **IgE response is preponderant**. About 10 per cent of persons have this tendency to overproduce IgE. The atopic antibody does not pass through the placenta.

Atopic sensitivity is caused by the overproduction of IgE antibodies. This is often associated with a deficiency of IgA. This association has led to the

suggestion that IgA deficiency may predispose a person to atopy. In normal individuals, the inhalant and ingestant antigens are dealt with by the IgA that line the respiratory and intestinal mucosa and therefore, they do not come into contact with potential IgE-producing cells. When IgA is deficient, the antigens cause massive stimulation of IgE-forming cells, leading to the overproduction of IgE.

Symptoms of atopy are caused by the release of pharmacologically active substances following the combination of the antigen and the cell-fixed IgE. The clinical expression of atopic reactions is usually determined by the portal of entry of the antigen—conjunctivitis, rhinitis, gastrointestinal symptoms and dermatitis following exposure through the eyes, respiratory tract, intestine or skin, respectively. Specific desensitisation (hyposensitisation) is often practised in the treatment of atopy.

Detection and Management

Several methods are used to detect Type I hypersensitivity:

- ***Skin test*** Small amounts of the suspected allergen are introduced by intradermal injection or superficial scratching. A wheal-and-flare within 30 minutes confirms allergy (Fig. 14.2). The local mast cells release histamine and other mediators.
- ***RAST*** Total levels of serum IgE can be determined **by** radioallergosorbent test. IgE can be estimated by fluorescent-labelled anti-IgE to detect IgE in the serum or by ELISA.
- ***Radioimmunosorbent test (RIST)*** This is another test that estimates the total circulating IgE antibodies.

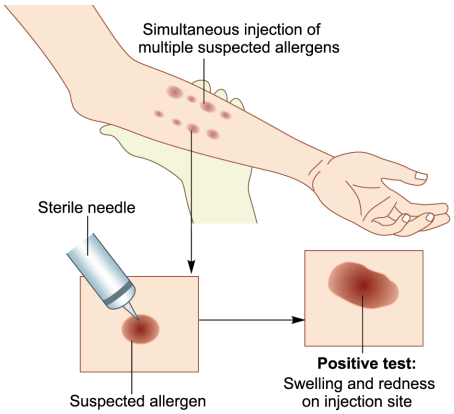


Fig. 14.2 Skin test to demonstrate immediate hypersensitivity

Treatment

Acute anaphylaxis or allergy can be managed by treating with epinephrine (adrenalin), antihistamines (block H_1 and H_2 receptors) and corticosteroids (reduce histamine levels). The doses are adjusted according to body weight.

TYPE II REACTIONS: CYTOLYTIC AND CYTOTOXIC REACTIONS

These reactions involve a combination of IgG (or rarely, IgM) antibodies which are directed towards antigenic determinants on the surface of cells, leading to complement activation, resulting in cytotoxic or cytolytic effects. Examples are the lysis of red cells caused by anti-erythrocyte antibodies in autoimmune anemias and hemolytic disease of the newborn. Alternatively, a free antigen or hapten may be absorbed on to cell surfaces. Subsequent reaction of the combined antigen or hapten with its corresponding antibody leads to cell damage. Many **drugs may behave in this manner, leading to the complement-mediated lysis of red cells, leucocytes and platelets, causing hemolytic anemia, agranulocytosis and thrombocytopenic purpura** (Fig. 14.3). Cytotoxic cells may be stimulated by the antigen–antibody complex. This is known as antibody-dependent cell cytotoxicity (ADCC).

An additional type of hypersensitivity, which is distinct from type II, is a stimulatory type sometimes known as **Type V hypersensitivity.** In this, IgG binds to the cell surface antigen receptors and has a stimulatory effect.This prevents the antigen ligand from binding the receptor or it mimics the ligand, thus affecting cell signalling. This mechanism is seen in Graves' disease and myasthenia gravis.

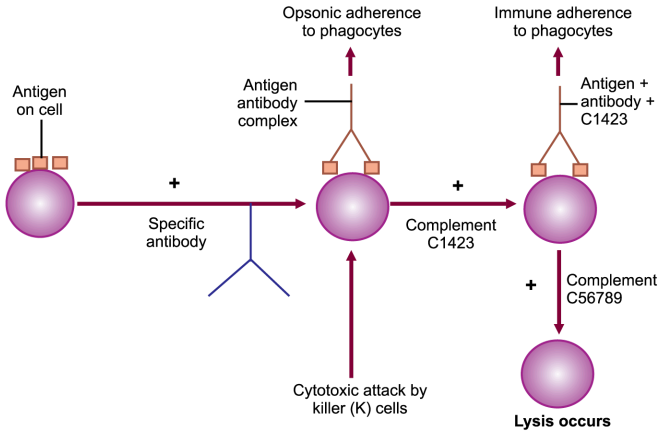


Fig. 14.3 Type II reaction (cytolytic and cytotoxic actions)

TYPE III REACTIONS: IMMUNE COMPLEX-MEDIATED HYPERSENSITIVITY

Antigen and antibody combine and take up the complement-forming and immune complex. These complexes get deposited and stimulate an inflammatory reaction. This forms the basis of Type III hypersensitivity.

Types

Serum sickness

This is a systemic form of Type III hypersensitivity. This type of hypersensitivity appears 7–12 days after a single injection of a high concentration of foreign serum such as the diphtheria antitoxin. The clinical syndrome consists of fever, lymphadenopathy, splenomegaly, arthritis, glomerulonephritis, endocarditis, vasculitis, urticarial rashes, abdominal pain, nausea and vomiting. The pathogenesis is the formation of **immune complexes** (consisting of the foreign serum and antibody to it, which reaches high-enough titres by 7–12 days; Fig. 14.4), which get deposited on the endothelial lining of blood vessels in various parts of the body, causing inflammatory infiltration. The nephritis and arthritis seen in these conditions may be caused by the deposition of immune complexes.

Serum sickness differs from other types of hypersensitivity reactions in that a single injection can serve both as the sensitising dose and the shocking dose. As heterologous serum injections are not used often now, the syndrome is currently more commonly seen following injection of

penicillin or other antibiotics.

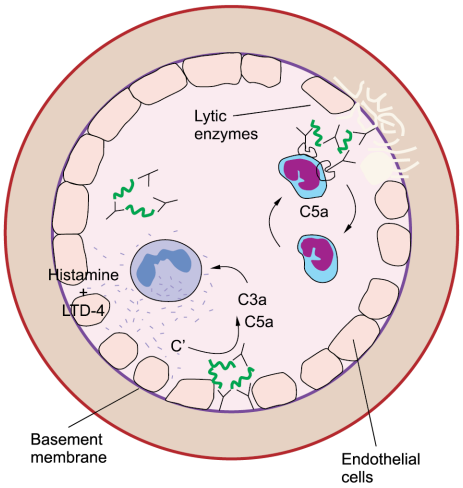


Fig. 14.4 Type III reaction (immune complex-mediated hypersensitivity (serum sickness)

Arthus reaction

This is a local manifestation of generalised hypersensitivity. The tissue damage is due to the formation of antigen–antibody precipitates causing complement activation and release of inflammatory molecules. This leads to increased vascular permeability and infiltration of the site with neutrophils. Leucocyte–platelet thrombi that reduce blood supply and lead to tissue necrosis are formed. The Arthus reaction can be passively transferred with sera containing precipitating antibodies (IgG or IgM) in high titres.

Arthus reaction forms a pathogenic component of many clinical syndromes. For example, intrapulmonary Arthus-like reaction to inhaled antigens, such as thermophilic actinomycetes from mouldy hay or grain, causes farmer's lung and other types of hypersensitivity pneumonitis.

TYPE IV REACTIONS: T CELL-MEDIATED HYPERSENSITIVITY (DELAYED TYPE)

Type IV hypersensitivity reactions (delayed hypersensitivity) are cell-mediated immune responses. These are typically provoked by intracellular microbial infections or hapten-like simple chemicals applied on the skin. The reaction evolves slowly and consists of a mixed cellular reaction involving lymphocytes and macrophages. It is induced by **sensitised T cells (T_{dth}, T_h1, T_h2, T_c)** which, on contact with the specific antigen, release cytokines that produce biological effects on leucocytes, macrophages and

tissue cells. **Delayed hypersensitivity cannot be passively transferred by serum but can be transferred by lymphocytes or the transfer factor**.

Types

Two types of delayed hypersensitivity are recognised: the tuberculin (infection) type and the contact dermatitis type.

1. Tuberculin (infection) type of delayed hypersensitivity

When a small dose of tuberculin is injected intradermally in an individual sensitised to tuberculoprotein by prior infection or immunisation, an indurated inflammatory reaction develops at the site within 48–72 hours. **In unsensitised individuals, the tuberculin injection provokes no response**. The tuberculin test therefore provides a useful indication of the state of delayed hypersensitivity (cell-mediated immunity) to the bacilli.

Tuberculin type hypersensitivity develops in several subacute or chronic bacterial, fungal, viral and parasitic infections and is generally seen when the pathogen is intracellular. A similar hypersensitivity is developed in allograft reactions and in many autoimmune diseases.

Cutaneous basophil hypersensitivity

A local reaction resembling the tuberculin response may be produced by the intradermal injection of some protein antigens. This is not a delayed hypersensitivity reaction as it can be passively transferred by serum. Its histology is different from that of the tuberculin response and is characterised by prominent basophil infiltration. This was formerly known as the Jones–Mote reaction but is now termed cutaneous basophil hypersensitivity. Its significance is not known.

2. Contact dermatitis

Delayed hypersensitivity sometimes results from skin contact with a variety of chemicals—metals such as nickel and chromium, simple chemicals like dyes, picryl chloride, dinitrochlorobenzene, drugs such as penicillin, plant allergen (parthenin from parthenium) and toiletries to which the individual has been sensitised earlier. Antibiotic ointments applied on patches of dermatitis frequently provoke sensitisation. The **substances involved are in themselves not antigenic but may acquire antigenicity on combination with skin proteins**. Sensitisation requires percutaneous absorption. As most of the substances involved are fat-soluble, passage along the sebaceous glands may be the method of entry of the allergens.

Mechanism

Langerhans' cells of the skin capture locally applied hapten, along with the modified tissue proteins, and migrate to the draining lymph nodes where they present the processed antigen along with MHC molecules to T cells. The sensitised T cells travel to the skin site, where on contacting the antigen, they release various lymphokines. T_h1 cells secrete IFN-γ and IL-2 which activate macrophages and other lymphocytes (Fig. 14.5). T_h2 cells release IL-4, IL-5, GM-CSF and other factors that lead to an influx of eosinophils and tissue damage. Activated T_c cells mediate the killing of target cells.

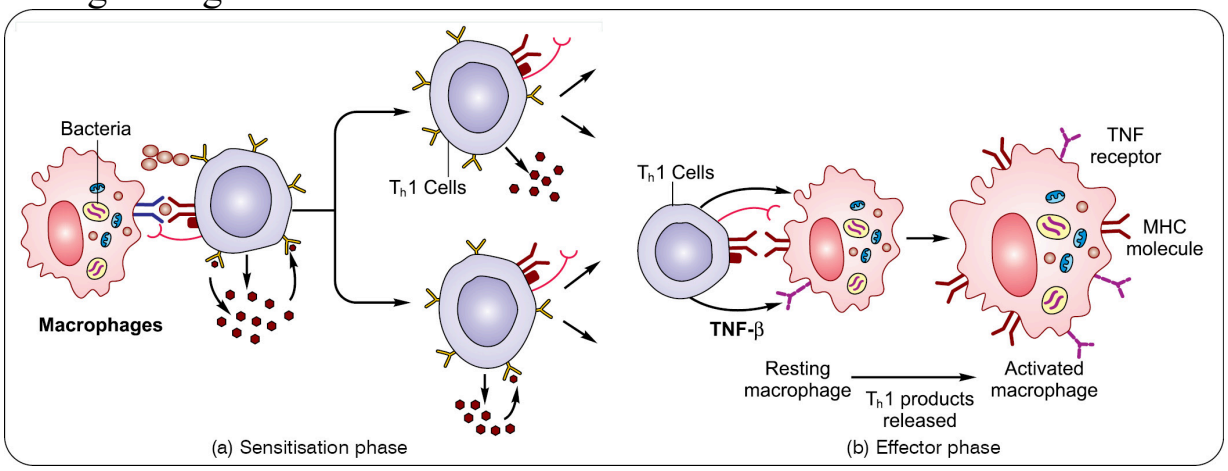


Fig. 14.5 (a) and (b) Mechanism of cell-mediated hypersensitivity (delayed type)

Symptoms

Contact with the allergen in a sensitised individual leads to '**contact dermatitis**', the lesions varying from macules and papules to vesicles that break down, leaving behind raw, weeping areas, typical of acute eczematous dermatitis.

- ***Detection using 'patch test'*** The allergen is applied to the skin under an adherent dressing. Sensitivity is indicated by the appearance of itchiness in 4–5 hours, and local reaction, which may vary from erythema to vesicle or blister formation, after 24–28 hours.
- ***Stimulatory hypersensitivity*** Here, the antibody activates receptor sites and enhances the activity of the cell. A few

examples are the action of long-acting thyroid stimulator (LATS), an antibody against some determinant on thyroid cells, which stimulates excessive secretion of the thyroid hormone, Graves' disease, Stevens-Johnson syndrome, sulphonamide-induced morbilliform rash, etc.

Schwartzman reaction

An intravenous injection of a substance followed by an intradermal injection of the same agent evokes a reaction at the site of the second inoculation, leading to **disseminated intravascular coagulation** and thrombohemorrhagic reaction at the local site of injection.

Clinical implications of Schwartzman reaction

- **Waterhouse–Friderichsen syndrome** It has been suggested that mechanisms similar to the Shwartzman reaction may operate in some clinical conditions such as the purpuric rashes of meningococcal septicemia and the acute hemorrhagic adrenal necrosis found in overwhelming infections.
- Lipopolysaccharide endotoxin and some gram-positive infections may also cause similar effects. *Staphylococcus aureus* may lead to **disseminated intravascular coagulation (DIC). Massive activation of complement by the alternative pathway, associated with the release of thromboxane A2, prostaglandins** and interleukins 1 and 6 by macrophages**,** may lead to the induction of cytokine TNF secretion by macrophages and peptidoglycan-mediated platelet aggregation, causing disseminated intravascular coagulation.

Key points

- Hypersensitivity reactions are pathologic processes that result from exaggerated (very vigorous) specific interactions between antigens (exogenous or endogenous) and either humoral antibodies or sensitised lymphocytes, resulting in tissue injury.
- It occurs only if the host has had an earlier contact with the antigen (allergen). The initial or sensitising dose sensitises the immune system, while subsequent contact (shocking dose)

with the same antigen (allergen) causes manifestations of hypersensitivity.

- Hypersensitivity reactions can be classified:
    - Based on the duration between exposure to antigen and the reaction, into immediate type hypersensitivity and delayed type hypersensitivity
    - Based on the component involved in the reaction, into antibody-mediated hypersensitivity and cell-mediated hypersensitivity
    - Based on Gell and Coombs classification, into Type I, Type II, Type III and Type IV
- Type I (immediate, anaphylactic), IgE-mediated hypersensitivity reactions occur within minutes of exposure to the antigen. The cross-linking of mast cell–bound IgE by the allergen causes the release of vasoactive substances (histamine, leukotrienes, prostaglandins, etc.), which produce inflammation. Diagnostic tests include total IgE levels, direct skin test, radioallergosorbent test, leucocyte histamine release test, provocative challenge and provocative food testing.
- Clinical conditions result from:
    - Type I reactions, which include systemic anaphylaxis, allergic extrinsic asthma, seasonal allergic rhinitis, reactions to the sting of an insect, some reactions to foods and drugs and some cases of urticaria.
    - Type II (cytotoxic) hypersensitivity reactions, where antibody binding to cell surface antigens is followed by antibody-dependent cell-mediated cytotoxicity by K cells, or complement-mediated lysis. Type II diseases include transfusion reactions, hemolytic disease of the newborn and certain drug allergies caused by cytotoxic T lymphocytes.
    - Type III (immune complex-mediated) hypersensitivity reactions, in which tissue damage occurs due to the activation of complement by immune complexes (through the classical pathway). It may result from persistent infection with streptococci, hepatitis B virus, malarial parasites and filarial worms; other examples include glomerulonephritis, alveolitis and certain autoimmune diseases.
    - Type IV (cell-mediated or delayed type) hypersensitivity reactions, in which symptoms appear at least 24–48 hours after exposure to the antigen. There is activation of T cells, release of lymphokines and subsequent influx of macrophages to the site. Allergic contact dermatitis (due to poison ivy) and the skin test for exposure to tubercle bacilli (tuberculin test) are examples.
- Stimulatory hypersensitivity reaction, in which the antibodies recognise and bind to the cell surface receptors, is a modified form of Type II reaction.

Questions

Essay

1. Classify hypersensitivity reactions with examples. Describe Type I hypersensitivity.

Short Answers

1. Anaphylaxis
2. Atopy
3. Arthus reaction
4. Serum sickness

5. Tuberculin type delayed hypersensitivity
6. Contact dermatitis
7. Delayed hypersensitivity
8. Mediators of anaphylaxis

CHAPTER 15

Immunodeficiency Diseases

Chapter navigation

- Primary immunodeficiency
- Secondary immunodeficiency

INTRODUCTION

The immune system is a complex multicomponent system in which one or more components might fail to function, leading to **immunodeficiency.** This leads to repeated microbial infections of varying severity in the host, and sometimes enhanced susceptibility to malignancies. Deficiencies of defence mechanisms may involve specific immune functions—humoral immunity, cell-mediated immunity or both—or non-specific mechanisms such as phagocytosis and complement activity.

Immunodeficiency is of two types:

i) **Primary immunodeficiency,** which results from abnormalities in the development of the immune system

ii) **Secondary immunodeficiency,** which is the consequence of disease, drugs, nutritional inadequacies and other processes that interfere with the proper functioning of the mature immune system

PRIMARY IMMUNODEFICIENCIES

The established types of primary immunodeficiency syndromes are listed in Table 15.1. Though primary deficiencies of specific immunity are classified as those affecting B or T cell responses, or both, it must be realised that there is considerable overlap due to the intimate interaction between the B cell and T cell systems. For instance, T cell deficiencies involving helper or suppressor T cells will have a profound effect on antibody response.

Table 15.1 Types of immunodeficiency syndromes

Disorders of specific immunity leading to immunodeficiency diseases	Specific defect
I. Humoral immunodeficiencies (B cell defects)	a) X-linked agammaglobulinemia b) Transient hypogammaglobulinemia of infancy c) Common variable immunodeficiency (late-onset hypogammaglobulinemia) d) Selective immunoglobulin deficiencies (IgA, IgM or IgG subclasses) e) Immunodeficiencies with hyper-IgM f) Transcobalamin II deficiency
II. Cellular immunodeficiencies (T cell defects)	a) Thymic hypoplasia (DiGeorge syndrome) b) Chronic mucocutaneous candidosis c) Purine nucleoside phosphorylase (PNP) deficiency
III. Combined immunodeficiency (B and T cell defects)	a) Cellular immunodeficiency with abnormal immunoglobulin synthesis (Nezelof syndrome) b) Ataxia telangiectasia c) Wiskott–Aldrich syndrome d) Immunodeficiency with thymoma e) Immunodeficiency with short-limbed dwarfism f) Episodic lymphopenia with lymphocytotoxin g) Severe combined immunodeficiencies 1. 'Swiss type' agammaglobulinemia 2. Reticular dysgenesis of de Vaal 3. Adenosine deaminase (ADA) deficiency
IV. Disorders of phagocytosis	a) Chronic granulomatous disease b) Myeloperoxidase deficiency c) Chediak–Higashi syndrome d) Leucocyte G6PD deficiency e) Job syndrome f) Tuftsin deficiency g) Lazy leucocyte syndrome h) Hyper-IgE syndrome i) Actin-binding protein deficiency j) Shwachman disease
V. Disorders of complement	a) Complement component deficiencies b) Complement inhibitor deficiencies

A. DISORDERS OF SPECIFIC IMMUNITY

I. Humoral Immunodeficiencies (Primary Immunodeficiencies)

i. X-linked agammaglobulinemia

This syndrome, which was described by Bruton (1952), is the first immunodeficiency disease to have been recognised. It is seen only in male infants.

Manifestations It presents as **recurrent serious infections** with pyogenic bacteria, particularly with pneumococci, streptococci, meningococci, *Pseudomonas* and *H. influenzae*. Patients respond normally to viral infections such as measles and chickenpox, though there have been reports of paralytic poliomyelitis and progressive encephalitis following immunisation with live virus vaccines or exposure to a wild virus. The disease is not apparent until about six months of age due to the passive protection by maternal antibodies.

> **As a general rule, live microbial vaccines should not be given to children with any type of primary immunodeficiency** (*Clinical case*).

Features

- **All classes of immunoglobulins are grossly depleted**; the IgG levels are less than a tenth, and IgA and IgM less than a hundredth of the normal level.
- Tonsils and adenoids are atrophic. Lymph node biopsy reveals depletion of cells of the bursa-dependent areas. Plasma cells and germinal centres are absent even after antigenic stimulation.
- There is marked decrease in the proportion of B cells in circulation. Antibody formation does not occur even after injection of antigens.
- **Cell-mediated immunity (CMI) is not affected.** Delayed hypersensitivity of tuberculin and contact dermatitis types can be demonstrated. Allograft rejection is normal. Arthritis, hemolytic anemia and atopic manifestations are frequently observed. However, the wheal-and-flare response of atopic hypersensitivity cannot be demonstrated.

Management This consists of the maintenance of an adequate level of immunoglobulins. Administration of 300 mg of gamma globulin per kg of body weight in three doses followed by monthly injections of 100 mg per kg are required. Commercial preparations of gamma globulin contain only traces of IgA and IgM; therefore, whole plasma infusions are used.

Clinical Case

A nine-month-old male infant was brought to the hospital with symptoms of fever and difficulty in breathing. The mother reported

two similar episodes in the previous two months. At the age of 12 months, the child was again brought in for an episode of measles, from which he recovered after treatment. At 18 months of age, it was observed that the boy's height and weight were not appropriate for his age. The child was the fourth of unrelated parents. Unlike the boy, his three sisters enjoyed good health and did not have any repeated infections.

Tests on the boy showed the serum immunoglobulin IgG to be less than a tenth and IgA and IgM to be less than a hundredth of the normal level. Lymph node biopsy revealed depletion of cells of the bursa-dependent areas. The child was diagnosed with Bruton's disease (X-linked agammaglobulinemia) and was treated with an initial administration of 300 mg of gamma globulin/kg body weight in 3 doses, followed by monthly injections of 100 mg/kg. The child responded well to treatment.

ii. Transient hypogammaglobulinemia of infancy

This is due to an abnormal delay in the initiation of IgG synthesis in some infants. Maternal IgG is slowly catabolised in the newborn and reaches a level of 200 mg per 100 mL by the second month of life. Ordinarily, an infant begins synthesising its own IgG by this age. A delay in the synthesis of IgG leads to immunodeficiency. Recurrent otitis media and respiratory infections are commonly seen. Spontaneous recovery occurs between 18 and 30 months of age. This disease may be found in infants of both sexes.

Management Treatment with gamma globulin may be required in some cases but it is not recommended prophylactically, as it may contribute to the prolongation of immunodeficiency by negative feedback inhibition of IgG synthesis.

iii. Common variable immunodeficiency

This common form of immunodeficiency is also known as **late-onset hypogammaglobulinemia** because it usually manifests only by 15–35 years of age. It is characterised by:

- Recurrent pyogenic infection
- Increased incidence of autoimmune disease
- Malabsorption
- Giardiasis
- Low level of total immunoglobulin
- Defective B cells in circulation—they fail to differentiate into plasma cells and secrete immunoglobulins.

Increased suppressor T cell and diminished helper T cell activity have been proposed as a cause of this disorder.

Management Treatment is by administration of gamma globulin preparations intramuscularly or intravenously.

iv. Selective immunoglobulin deficiencies

In these conditions, there is selective deficiency of one or more immunoglobulin classes, while the others remain normal or elevated. These '**dysgammaglobulinemias**' are common and have been reported in about one per cent of all patients with recurrent infection.

- **Isolated IgA deficiency** is the most common condition in this group, with a reported incidence of about 0.2 per cent in normal populations. The patients exhibit increased susceptibility to respiratory infection and steatorrhea. IgA deficiency is often accompanied by atopic disorders. Anti-IgA antibodies are present in many of these patients.
- **Selective IgM** deficiency has been found to be associated with septicemia.
- **Deficiencies of IgG subclasses** have been observed in relation with chronic progressive bronchiectasis.

Management Preventative antibiotics may be used to help diminish the frequency of recurrent infections. Individuals with IgA deficiency often require a longer course of antibiotics for infections to clear up.

v. Immunodeficiencies with hyper-IgM

In this group of immunodeficiencies, some of which are X-linked and some

inherited as autosomal recessive, low IgA and IgG levels are seen with elevated IgM. Patients show **enhanced susceptibility to infections and autoimmune processes such as thrombocytopenia, neutropenia, hemolytic anemia and renal lesions**. Some patients develop malignant infiltration with IgM-producing cells. Elevated IgM level with immunodeficiency is sometimes seen in congenital rubella.

Management Intravenous immunoglobulin (IVIG) therapy has reportedly decreased the frequency of severe and lower respiratory tract infections significantly.

vi. Transcobalamin II deficiency

In this disorder, which is inherited as an autosomal recessive trait, patients show the metabolic effects of vitamin B12 deficiency including **megaloblastic anemia** and **intestinal villous atrophy**. The associated immunological defects are (i) depleted plasma cells, (ii) diminished immunoglobulin levels and (iii) impaired phagocytosis.

Management Treatment with vitamin B12 has been reported to restore hematopoietic, gastrointestinal and B cell functions but not phagocytic activity.

II. Cellular Immunodeficiencies

i. Thymic hypoplasia (DiGeorge syndrome)

This is a developmental defect involving the endodermal derivatives of the third and fourth pharyngeal pouches. It leads to aplasia or hypoplasia of the thymus and parathyroid glands. It does not appear to be hereditary and does not show a familial incidence. It is probably due to some intrauterine infection or other complications. It is usually associated with Fallot's tetralogy and other anomalies of the heart and the great vessels, and a characteristic facial appearance. Neonatal tetany is present. Patients who survive the neonatal period show enhanced susceptibility to viral, fungal and bacterial infections, which ultimately prove fatal.

The immunodeficiency primarily involves cell-mediated immunity. **The thymus-dependent areas of the lymph nodes and spleen are depleted of lymphocytes**. Circulating T cells are reduced in number. Delayed hypersensitivity and graft rejection are depressed. The humoral immune

mechanism is largely unaffected.

Management Transplantation of fetal thymus tissue has been reported to restore the immunological function.

ii. Chronic mucocutaneous candidosis

This constitutes an abnormal immunological response to *Candida albicans*. Patients develop severe chronic candidosis of the mucosa, skin and nails. They do not show increased susceptibility to other infections but often have endocrinopathies. CMI to candida is deficient. Delayed hypersensitivity to candida antigens is absent but circulating antibodies to them are found in high titres. Intracellular killing of candida is defective.

Management Transfer factor therapy, along with amphotericin B, has been reported to be effective.

iii. Purine nucleoside phosphorylase (PNP) deficiency

The enzyme purine nucleoside phosphorylase is involved in the sequential degradation of purines to hypoxanthine and finally, to uric acid. Patients who have PNP deficiency as an autosomal recessive inherited trait show decreased CMI and recurrent or chronic infection. They usually present with hypoplastic anemia and recurrent pneumonia, diarrhea and candidosis. Due to defects in purine degradation, serum uric acid is low and may be diagnostic.

Management Allogeneic hematopoietic stem cell transplantation (HSCT) may be the choice of treatment to restore immunological functions.

III. Combined Immunodeficiencies

i. Cellular immunodeficiency with abnormal immunoglobulin synthesis (Nezelof syndrome)

The term Nezelof syndrome has been rather loosely applied to a group of disorders, probably of varied origin, where depressed CMI is associated with selectively elevated, decreased or normal levels of immunoglobulin. The consistent features are a marked deficiency of T cell immunity and varying degrees of deficiency of B cell immunity. Patients are susceptible to recurrent fungal, bacterial, viral and protozoal diseases. Thymic dysplasia occurs with lymphoid depletion. Autoimmune processes such as hemolytic

anemia are common. In spite of normal levels of immunoglobulins, antigenic stimuli do not induce antibody formation.

Management Histocompatible bone marrow transplantation, transfer factor and thymus transplantation have been used for treatment, with success in some cases. Adequate antimicrobial therapy is essential.

ii. Ataxia telangiectasia

This is a hereditary condition transmitted in the autosomal recessive mode, where combined immunodeficiency is associated with cerebellar ataxia, telangiectasia, ovarian dysgenesis and chromosomal abnormalities. Ataxia and choreoathetoid movements are usually noticed in infancy. Telangiectasia involving the conjunctiva, face and other parts of the body usually appears at five or six years of age. Death occurs due to sinopulmonary infection early in life, or malignancy in the second or third decade. The majority of patients lack serum and secretory IgA (some have antibodies to IgA). IgE deficiency is also frequent. CMI is also defective, resulting in the impairment of delayed hypersensitivity and graft rejection.

Management Transfer factor therapy and fetal thymus transplants have been tried with some amount of success.

iii. Wiskott–Aldrich syndrome

This is an X-linked disease characterised by eczema, thrombocytopenic purpura and recurrent infections. Affected boys rarely survive the first decade of life, death being due to infection, hemorrhage or lymphoreticular malignancy.

CMI undergoes progressive deterioration associated with cellular depletion of the thymus and the paracortical areas of the lymph nodes. Serum IgM level is low but IgG and IgA levels are normal or elevated. Isohemagglutinins are absent in the serum. The humoral defect appears to be a specific inability to respond to polysaccharide antigens.

Management Bone marrow transplantation and transfer factor therapy have been found to be beneficial.

iv. Immunodeficiency with thymoma

This syndrome, occurring usually in adults, consists of a benign thymic tumour, impaired cell-mediated immunity and agammaglobulinemia. It is

frequently accompanied by aplastic anemia. This is of historical importance as an experiment of nature which suggested the immunological function of the thymus.

- ***Immunodeficiency with short-limbed dwarfism*** The features of this condition are a distinctive form of short-limbed dwarfism, ectodermal dysplasia, thymic defects and enhanced susceptibility to infection. These defects are apparently inherited as autosomal recessives.
- ***Episodic lymphopenia with lymphocytotoxin*** In this syndrome, there occurs an episodic but profound depression of T cell function by the action of a circulating complement-dependent lymphocytotoxin. The toxin appears to be an anti-lymphocyte antibody. The patients lack 'immunological memory' so the secondary antibody response is abolished. The disease is familial.

v. Severe combined immunodeficiencies (SCID)

These include many syndromes with severe deficiency of both humoral and CMI response. They are inherited in the autosomal recessive mode and the primary defects are at the level of the early precursors of immunocompetent cells in the fetal liver and bone marrow.

- ***Swiss-type agammaglobulinemia*** The basic defect is presumed to be at the level of the lymphoid stem cell. (In 1958, Swiss workers reported agammaglobulinemia with lymphocytopenia and severe defect in CMI.)
- ***Reticular dysgenesis of de Vaal*** Here, the defect is at the level of the multipotent hemopoietic stem cell. As a result, there is total failure of myelopoiesis, leading to lymphopenia, neutropenia, thrombocytopenia, anemia and bone marrow aplasia. The most serious form of this condition is invariably fatal in the first week of life.
- ***Adenosine deaminase (ADA) deficiency*** This is associated with an enzyme deficiency. ADA catalyses the conversion of adenosine to inosine in the purine metabolic pathway. How this

deficiency causes immunological impairment is not clear. The range of immunodeficiency varies from complete absence to mild abnormalities in B and T cell function. The condition is associated with chondrocyte abnormalities which can be discerned radiologically.

B. DISORDERS OF COMPLEMENT

I. Complement Component Deficiencies

Genetic deficiencies have been detected for almost all the complement components in human beings. The defects are transmitted as autosomal recessive traits. Complement component deficiencies have been frequently associated with systemic lupus erythematosus. Recurrent pyogenic infections were found associated with C3 deficiency and neisserial infections with deficiency of C6, C7 and C8.

II. Complement Inhibitor Deficiencies

Hereditary angioneurotic edema is due to a genetic deficiency of the C1 inhibitor. This relatively common defect is transmitted as an autosomal dominant trait. The rare deficiency of the C3b inactivator has been associated with chronic recurrent pyogenic lesions.

Management Androgens, aminocaproic acid and its analogue tranexamic acid have been found useful in the management of this condition.

C. DISORDERS OF PHAGOCYTOSIS

Phagocytosis may be impaired by:

i) **Intrinsic disorders,** which may be due to defects within the phagocytic cell, such as enzyme deficiencies.

ii) **Extrinsic disorders,** which may be due to a deficiency of an opsonic antibody, complement or other factors promoting phagocytosis. They could also be caused by the effects of drugs or anti-neutrophil autoantibodies. Phagocytic dysfunction leads to increased susceptibility to infection, ranging from mild recurrent skin infections to overwhelming systemic infection.

i. Chronic granulomatous disease

This familial disease manifests as recurrent infection with low-grade pathogens, starting early in life. Chronic suppurative granulomatous lesions develop in the skin and lymph nodes, along with hepatosplenomegaly, progressive infiltration of the lungs and granulomatous septic osteomyelitis. Humoral and cellular immune response are normal. The progress is chronic and the outcome fatal. The bacteria involved in the recurrent infections are catalase-positive pyogenic pathogens such as staphylococci and coliforms. Catalase-negative pathogens such as streptococci and pneumococci are handled normally. The host's leucocytes are unable to kill catalase-positive bacteria following phagocytosis, as a result of which, the bacteria multiply intracellularly. Diminished bactericidal capacity of the phagocytic cells is associated with a decrease of some metabolic processes. **Diminished H_2O_2 production appears to be the main reason for the bactericidal defect**. The leucocytes do not undergo degranulation following phagocytosis. Delayed granule rupture and defective release of myeloperoxidase also contribute to inefficient bactericidal activity.

The disease shows two types of inheritance: the more common, X-linked type seen in boys and the rare autosomal recessive type seen in girls.

Nitroblue tetrazolium test Leucocytes from the patients fail to reduce nitroblue tetrazolium (NBT) during phagocytosis. This property is used as a screening method (NBT test) for the diagnosis of chronic granulomatous disease.

ii. Myeloperoxidase deficiency

In this rare disease, leucocytes have reduced myeloperoxidase. Patients are particularly prone to *Candida albicans* infection.

iii. Chediak–Higashi syndrome

This is a genetic disorder characterised by decreased pigmentation of the skin, eyes and hair, photophobia, nystagmus and giant peroxidase-positive inclusions in the cytoplasm of leucocytes. The inclusions may be the result of autophagocytic activity. The leucocytes possess diminished phagocytic activity. Patients suffer from frequent and severe pyogenic infections.

iv. Leucocyte G6PD deficiency

In this rare disease, leucocytes are deficient in glucose-6-phosphate dehydrogenase and show diminished bactericidal activity after phagocytosis. The condition resembles chronic granulomatous disease in reduced myeloperoxidase activity and susceptibility to microbial agents; however, the NBT test may be normal.

v. Job syndrome

This is characterised by multiple, large, 'cold' staphylococcal abscesses containing large quantities of pus that develop repeatedly on the skin and in various organs, with little inflammatory response. Atopic eczema, chronic nasal discharge and otitis media are common features. Serum immunoglobulins are normal, except for elevated IgE. The pathogenesis of the syndrome is not clear but it is probably a primary defect in phagocytic function.

vi. Tuftsin deficiency

A leucokinin capable of stimulating phagocytosis, discovered at Tufts University, Boston, has been designated the name 'tuftsin'. Chemically, it is a small tetrapeptide (Thr-Lys-Pro-Arg). Patients with tuftsin deficiency have been reported to be prone to local and systemic bacterial infections.

vii. Lazy leucocyte syndrome

The basic defect here is in chemotaxis and neutrophil mobility. The bone marrow has a normal number of neutrophils but there is peripheral neutropenia with poor leucocyte response to chemical and inflammatory stimulation. Patients show increased susceptibility to bacterial infection with recurrent stomatitis, gingivitis and otitis.

viii. Hyper-IgE syndrome

In this disease, which is seen in both sexes, patients have early-onset eczema and recurrent bacterial infections such as abscess, pneumonia and secondary infection of eczema. The organisms responsible include *Staphylococcus aureus* and *Streptococcus pyogenes.* Cellular and humoral immune mechanisms are normal but serum IgE levels are usually more than ten times the normal level.

ix. Actin-binding protein deficiency

Frequent infection and slow mobility of leucocytes results from the defective actin-binding protein in these patients.

x. Shwachman's disease

In this condition, frequent infections are found together with decreased neutrophil mobility, pancreatic malfunction and bone abnormalities.

SECONDARY IMMUNODEFICIENCIES

Secondary immunodeficiencies are much more common than primary deficiencies.

Agent-induced immunodeficiency A variety of factors such as malnutrition, malignancy, infection, metabolic disorders and cytotoxic drugs may lead to deficits in specific and non-specific immunity.

Humoral Deficiency

B cells are depleted, as in lymphoid malignancy, particularly in chronic lymphatic leukemia.

- Immunoglobulin catabolism is increased, as seen in nephrotic syndrome.
- Excessive loss of serum protein occurs, as seen in exfoliative skin disease and in protein-losing enteropathies.
- Excessive production of abnormal immunoglobulins occurs, as in multiple myeloma.

Cell-Mediated Immunity Deficiency

CMI is depressed in lymphoreticular malignancies, as in Hodgkin's disease.

- Lymph circulation is obstructed or lymphorrhea occurs.
- The thymus-dependent areas of the lymph nodes are infiltrated with non-lymphoid cells, as in lepromatous leprosy.
- CMI is suppressed transiently, following certain viral infections such as measles.

Nutritional deprivation affects both types of immune response adversely. Ageing also causes waning in the efficiency of acquired immunity.

Immunodeficiency follows the intentional or unintentional administration of immunosuppressive agents.

Acquired immunodeficiency syndrome

A severe form of immunodeficiency occurs due to infection by the human immunodeficiency virus (HIV 1 and 2), which is transmitted through sexual contact; from mother to child or through infected blood transfusion. There is cell-mediated immune deficiency initially followed by both humoral and CMI deficiency leading to death (*See* Chapter 53).

LABORATORY METHODS TO DETECT IMMUNODEFICIENCY DISORDERS

1. B cells

Humoral immunodeficiency is detected by estimating the B cells that may be absent in certain immune disorders associated with antibodies. This can be done by flow cytometry, which can identify B cells.

2. T cells

The simplest test to evaluate possible decrease or absence of T cells is a complete blood count (CBC) and differential to establish the total blood (absolute) lymphocyte count.

3. Severe Combined Immune Deficiency (SCID)

This condition can be evaluated by mutation analysis, which is the most accurate means to establish the definitive diagnosis. Patients can be tested for certain typical mutations [e.g., in the IL-2 receptor gamma (*IL-2RG* or *IL-2R*γ) gene].

4. Complement

The standard screening test for deficiencies in the complement system is the total hemolytic complement assay or CH50. This is estimated in terms of the serum dilution required to lyse 50% of antibody-coated RBCs. This test (called CH50) detects complement component deficiencies in the classical complement pathway but does not indicate which component is abnormal. A similar test (AH50) can be done to detect complement deficiencies in the alternative pathway.

5. Complete blood count (CBC)

- ***Peripheral blood smear*** Granulocytes may have morphologic abnormalities.
    - **Neutropenia** (absolute neutrophil count < 1,200 cells/μL) may be congenital or cyclic or may occur in aplastic anemia.
    - **Lymphopenia** (lymphocytes < 2,000/μL at birth, < 4,500/μL at age 9 months, or < 1,000/μL in older children or adults) suggests a T cell disorder because 70% of circulating lymphocytes are T cells.
    - **Leucocytosis** that persists between infections may occur in leucocyte adhesion deficiency.
    - **Thrombocytopenia** in male infants suggests Wiskott-Aldrich syndrome.
- ***Quantitative serum Ig levels*** Low serum levels of IgG, IgM or IgA suggest antibody deficiency. An IgG level < 200 mg/dL usually indicates significant antibody deficiency and is also seen in nephrotic syndrome or protein-losing enteropathies.
- ***IgM antibody*** This is assessed by measuring isohemagglutinin titres (anti-A and anti-B). Antibodies to blood groups A and B and to some bacterial polysaccharides are selectively deficient in certain disorders (e.g., Wiskott–Aldrich syndrome and complete IgG2 deficiency). People with blood type AB have natural antibodies at a titre of ≥ 1:8 (anti-A) or ≥ 1:4 (anti-B) (except infants < 6 months).
- ***IgG antibody*** This can be assessed in immunised patients by measuring antibody titres before and after the administration of vaccine antigens, e.g., *Haemophilus influenzae* type B, tetanus, diphtheria, conjugated or nonconjugated pneumococcal and meningococcal antigens. Less than two-fold increase in titre at 2 to 3 weeks suggests antibody deficiency.
- ***Phagocytic cell deficiency*** CD15 and CD18 are measured by flow cytometry, and neutrophil chemotaxis is tested. A flow cytometric oxidative (respiratory) burst assay measured by

nitroblue tetrazolium (NBT) can detect whether oxygen radicals are produced during phagocytosis. This is absent in chronic granulomatous disease.

Key points

- Immunodeficiency diseases are conditions in which the body's defence mechanisms are impaired, resulting in repeated microbial infections of variable severity and, sometimes, enhanced susceptibility to malignancies.
- Deficiencies of defence mechanisms may involve specific immune functions, namely humoral and/or cell-mediated immunity, or non-specific mechanisms (phagocytosis, complement), which augment specific immune processes. Immunodeficiencies may be primary or secondary.
- Primary immunodeficiencies result from abnormalities in the development of immune mechanisms and comprise the following:
    - Disorders of specific immunity, such as B cell defects or humoral immunodeficiencies (X-linked agammaglobulinemia, transient hypogammaglobulinemia of infancy, common variable immunodeficiency and selective immunoglobulin deficiencies)
    - T cell defects or cellular immunodeficiencies (thymic hypoplasia and chronic mucocutaneous candidosis)
    - Combined B and T cell defects or combined immunodeficiencies (cellular immunodeficiency with abnormal immunoglobulin synthesis, ataxia telangiectasia, Wiskott–Aldrich syndrome and severe combined immunodeficiencies)
    - Disorders of complement (deficiencies of complement components and deficiencies of complement inhibitor)
    - Disorders of phagocytosis (chronic granulomatous disease, myeloperoxidase deficiency, Chediak–Higashi syndrome, leucocyte glucose-6-phosphate deficiency, Job syndrome and tuftsin deficiency)
- Secondary immunodeficiencies occur due to certain infections (acquired immunodeficiency syndrome), nutritional deprivation (kwashiorkor), use of certain drugs (immunosuppressive agents, corticosteroids), metabolic disorders (protein losing enteropathies) and malignancies (chronic lymphocytic leukemia, multiple myeloma and Hodgkin's disease). Secondary immunodeficiencies are more common than primary immunodeficiencies.

Questions

Short Answers

1. Primary immunodeficiency disorders
2. Secondary immunodeficiencies
3. Humoral immunodeficiency disorders

4. Cellular immunodeficiencies
5. Combined B and T cell defects
6. Chronic granulomatous disease
7. Chronic mucocutaneous candidosis
8. Nezelof syndrome
9. Severe combined immunodeficiencies
10. Disorders of phagocytosis

Part II: Immunology

CHAPTER 16

Autoimmunity and Immunohematology

Chapter navigation

- Autoimmune diseases
- Immunohematology

Autoimmune disorders are those in which the immune system produces antibodies (known as autoantibodies) to endogenous (self) antigens, resulting in tissue injury and leading to disease. The condition was known as horror autotoxicus (Paul Erlich, 1901). Autoimmune disorders occur due to failure of **tolerance of the immune system to protect against self-reactive lymphocytes.**

IMMUNOLOGICAL TOLERANCE

Several innate mechanisms protect the body's cells from destruction by its own immune cells. This is referred to as tolerance.

- This may be central tolerance, which deletes T and B cell clones if they have any self-antigen-recognising receptors.
- As a back-up, in case of failure of central tolerance to delete these T and B cells, the secondary lymphoid organs render these cells inactive. This is known as peripheral tolerance.

The failure of these two mechanisms leads to autoimmunity.

MECHANISMS OF AUTOIMMUNITY

Inappropriate immune response to self-antigens can occur by the following

mechanisms:

1. ***Antigenic alterations*** Cells or tissues may undergo antigenic alterations as a result of physical, chemical and biological influences. Such altered antigens or '**neoantigens**' may elicit an immune response. Neoantigens can arise by physical agents such as irradiation, photosensitivity and cold allergy.

    - Drug-induced anemias, leucopenias and thrombocytopenias often have an autoimmune basis.
    - Infectious microorganisms, particularly viruses and other intracellular pathogens, may induce alteration of cell antigens. Viral infections, such as infectious mononucleosis, are known to often precede autoimmune disease. Bacterial enzymes also induce alteration of cell antigens. Neuraminidases formed by myxoviruses and several bacteria act on erythrocytes, thereby releasing the T antigen. Neoantigens may also arise by mutation. Such mutant cells may be immunogenic.

2. ***Sequestered antigens*** Certain self-antigens are present in closed systems and are not accessible to the immune apparatus. These are known as sequestered antigens. Some examples of sequestered antigens are:

    - The lens antigen of the eye is a sequestered antigen. The lens protein is enclosed in its capsule and does not circulate in blood. Hence immunological tolerance against this antigen is not established during fetal life. When the antigen leaks out following a penetrating injury, it may induce an immune response, causing damage to the lens of the other eye.
    - Since spermatozoa develop only with puberty, the sperm antigen cannot induce tolerance during fetal life. This antigen is, therefore, not recognised as self and is immunogenic when it enters circulation. This is believed to explain the pathogenesis of orchitis following mumps. The virus damages the basement membrane of seminiferous tubules, leading to the leakage of

sperms and the initiation of an immune response, resulting in orchitis.

3. ***Cross-reacting foreign antigens*** The fortuitous similarity between some foreign and self-antigens is the basis of the **'cross-reacting antigen' theory** of autoimmunity. Organ-specific antigens are present in several species. The injection of heterologous organ-specific antigens may induce an immune response, damaging the particular organ or tissue in the host.

    - Antirabies neural vaccine of infected sheep brain tissue used for immunisation results in neurological injury, which was, in the past, a complication of such a vaccination in humans. Its injection elicits an immune response against sheep brain antigens which may damage the individual's nerve tissue due to the cross-reaction between human and sheep brain antigens.
    - Streptococcal M proteins and the heart muscle share antigenic characteristics. The immune response induced by repeated streptococcal infection can therefore damage the heart. Nephritogenic strains of streptococci possess antigens found in the renal glomeruli. Infection with such strains may lead to glomerulonephritis due to antigenic sharing.

4. ***Molecular mimicry*** A related type of autoimmunisation is 'molecular mimicry', which is due to the presence in some infecting microorganisms and self-antigens of **epitopes with identical peptide sequences** (instead of similarities in 'cross-reactions'). **Epitope spreading** is a phenomenon that occurs when the self-cryptic antigenic epitope of self-antigens is displayed (exposed) following tissue damage due to local inflammatory or cytokine action. Examples of such homologous sequences are seen in arthritogenic *Shigella flexneri* and HLA-B27, *Mycobacterium tuberculosis* and joint membranes, Coxsackie B and myocardium. Epitope spreading leads to the progression and chronicity of the initial (acute) phase of the autoimmune disease. **Another related phenomenon is bystander activation, in which, T cells specific to antigen Y are activated in**

response to antigen X. This is sometimes seen in viral infections that precede autoimmunity. Molecular mimicry, bystander activation and epitope spreading are three mechanisms that can initiate immunoreactivity, leading to autoimmune disease.

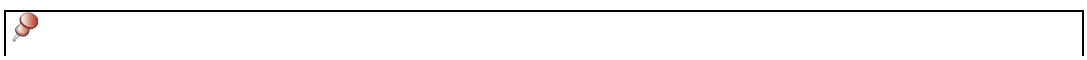
5. ***Polyclonal B cell activation*** In this hypothesis, while an antigen generally activates only its corresponding B cell, certain stimuli non-specifically turn on multiple B cell clones. Such stimuli include:

    - Chemicals (e.g., 2-mercaptoethanol) and bacterial products (e.g., PPD and lipopolysaccharide),
    - Enzymes (trypsin) and antibiotics (nystatin) and
    - Infections with some bacteria (mycoplasma), viruses (EB virus) and parasites (malaria). Multiple non-specific antibodies are formed during some infectious diseases such as anti-human erythrocyte cold antibodies in mycoplasma pneumonia and anti-sheep erythrocyte antibodies in infectious mononucleosis. These polyclonal antibodies are IgM in nature, similar to the 'natural antibodies' produced by $CD5^+$ B cells.

6. ***Forbidden clones*** The breakdown of immunological homeostasis may lead to cessation of tolerance and the emergence of forbidden clones of immunocompetent cells capable of mounting immune response against self-antigens. Autoimmunisation may result when tolerance to a self-antigen is abrogated, as for instance by the injection of the **self-antigen** with **Freund's adjuvant.**

7. ***Altered T or B cell function*** Enhanced helper T cell and decreased suppressor T cell functions have been suggested as causes of autoimmunity. Defects in the thymus, in stem cell development and macrophage function have also been postulated as causes.

Defects in the idiotype–anti-idiotype network have also been said to lead to autoimmunity. Genetic factors such as defective *Ir* or immunoglobulin genes have also been postulated. In spite of so many different possible mechanisms proposed, their actual role in autoimmunity, if any, has not been established.



Features of autoimmune disorder

- Elevated levels of immunoglobulins
- Demonstrable autoantibodies
- Deposition of immunoglobulins or their derivatives at sites of lesion such as renal glomeruli
- Accumulation of lymphocytes and plasma cells at the sites of lesion
- Benefit from corticosteroid or other immunosuppressive therapy
- The occurrence of more than one type of autoimmune lesion in an individual
- A genetic predisposition towards autoimmunity
- Higher incidence among females
- Chronicity, usually non-reversible

Classification of Autoimmune Diseases

Based on the site of involvement and nature of the lesions, autoimmune diseases may be classified as:

A. Organ-specific (hemocytolytic, localised)

B. Systemic (non-organ-specific)

A. Organ-Specific Autoimmune Diseases

1. Hemocytolytic autoimmune diseases (affecting the RBCs)

- ***Autoimmune hemolytic anemias*** Autoantibodies against erythrocytes are demonstrable in this condition. Serologically, two groups of autoimmune anemias can be distinguished, characterised by 'cold' and 'warm' antibodies, respectively.
    - **Cold autoantibodies** are, generally, complete agglutinating antibodies belonging to the IgM class and agglutinate erythrocytes at 4°C but not at 37°C. This condition, which used to frequently accompany syphilitic infection, is seldom seen nowadays. Cold agglutinins are also seen in primary atypical pneumonia, trypanosomiasis and black water fever.
    - **Warm autoantibodies** are generally incomplete, non-agglutinating antibodies, usually belonging to the IgG class and frequently seen in patients taking certain drugs such as sulphonamides, antibiotics and alpha-methyldopa.

      In autoimmune anemias, the red cells coated with

antibodies are prematurely destroyed in the spleen and liver. Complement-dependent intravascular hemolysis appears to be a rare event.

- ***Autoimmune thrombocytopenia*** Autoantibodies directed against platelets occur in idiopathic thrombocytopenic purpura. Sedormid purpura is an instance of immune response against drug-induced neoantigens on platelets.
- ***Autoimmune leucopenia*** Non-agglutinating anti-leucocyte antibodies can be demonstrated in the serum of patients with systemic lupus erythematosus and rheumatoid arthritis.

2. Localised (organ-specific) autoimmune diseases

- ***Hashimoto's disease (lymphadenoid goitre)*** Hashimoto's disease occurs more frequently in females and is associated with the enlargement of the thyroid gland and symptoms of hypothyroidism or frank myxedema. Histologically, the glandular structure is replaced by lymphoid tissue consisting of lymphocytes, histiocytes and plasma cells. Antibodies with different specificities have been found in this condition. They include antibodies that react with thyroglobulin, a second acinar colloid, microsomal antigen and a thyroid cell-surface component.
- ***Thyrotoxicosis (Graves' disease)*** Most patients with thyrotoxicosis possess antibodies to thyroglobulin. Lymphocytic infiltration is common in thyrotoxic glands. The immunological basis of thyrotoxicosis is supported by the identification of the '**long-acting thyroid stimulator**' **(LATS),** which is an IgG antibody to the thyroid membrane antigen. Combination of LATS with the surface membrane of thyroid cells seems to stimulate excessive hormone secretion.
- ***Addison's disease*** The immunological basis of Addison's disease is suggested by lymphocytic infiltration of the adrenal glands and the presence of circulating antibodies directed against the cells of the zona glomerulosa.

- ***Autoimmune orchitis*** Lymphocytic infiltration of the testes and circulating antibodies to the sperms and germinal cells can be demonstrated in this condition. This condition sometimes follows mumps orchitis.
- ***Myasthenia gravis*** In this disease, there is abnormal fatigability of muscles due to malfunction of the myoneural junction. An antibody against the acetylcholine receptor on myoneural junctions of striated muscles is present in these patients. This prevents acetylcholine from combining with its receptor, and impairs muscular contraction. The thymus shows lymphoid hyperplasia and numerous germinal centres. Infants born to affected mothers show symptoms of the disease but recover spontaneously by the age of two months, coinciding with the disappearance of maternal antibodies. This suggests that the pathogenic factor in neonatal myasthenia may be the autoantibody passively acquired from the mother.
- ***Autoimmune diseases of the eye*** Two types of autoimmune disease are seen in the eye. Cataract surgery sometimes leads to intraocular inflammation caused by autoimmune response to the lens protein. This is known as **phacoanaphylaxis**. Perforating injuries of the eye, particularly those involving the iris or ciliary bodies, are often followed by **sympathetic ophthalmia** in the opposite eye.
- ***Pernicious anemia*** Two types of autoantibodies are present in this condition. The first is directed against the parietal cells of the gastric mucosa. This is believed to cause achlorhydria and atrophic gastritis. The second is directed against the intrinsic factor and prevents absorption of vitamin B12 either by blocking its attachment to the gastric intrinsic factor or by binding to the B_{12} intrinsic factor complex and interfering with its uptake by the intestinal mucosa.
- ***Autoimmune diseases of the nervous system*** The 'neuroparalytic accidents' following rabies vaccination represent injury to the nervous system by the immune response against the sheep nerve tissue in the vaccine, which cross-reacts with human

nerve tissue. Idiopathic polyneuritis (Guillain–Barré syndrome) is considered an autoimmune response against the peripheral nervous tissue.

- ***Autoimmune diseases of the skin*** Three serious diseases of the skin are considered to have an autoimmune basis. Pemphigus vulgaris may be caused by an antibody to the intercellular adhesion protein desmoglein. In bullous pemphigoid, antibodies directed against the dermal epithelial junction have been demonstrated. Specific antibodies in dermatitis herpetiformis have not been identified.

B. Systemic (Non-organ-Specific) Autoimmune Diseases

This group includes conditions characterised by immune response against a variety of self-antigens and damage to several organs and tissue systems.

- ***Systemic lupus erythematosus (SLE)*** This is a chronic, multisystem disease with remissions and exacerbations, terminating fatally. Patients have a variety of autoantibodies directed against cell nuclei, intracytoplasmic cell constituents, immunoglobulins, thyroid and other organ-specific antigens. Biological false positive reaction is seen in standard tests for syphilis.

The LE cell is a neutrophil containing a large, pale, homogeneous body (**LE body**) almost filling the cytoplasm. The LE body is the immunologically damaged nucleus of a leucocyte. Sometimes, instead of being intracellular, the LE body can be seen free, surrounded by a rosette of neutrophils.

Immunofluorescent tests for antinuclear antibodies (ANA) show up different patterns of staining, such as homogeneous (diffuse), peripheral (outline), speckled and nucleolar (*Clinical case*). They are sensitive but not specific for SLE, as they may be positive in many other autoimmune conditions, viral infections, chronic inflammatory processes, etc.

Anti-DNA antibodies are tested by RIA or ELISA. Three major types of these antibodies are seen—those reacting with single-stranded **(ss)** DNA, those with double-stranded **(ds)** (DNA) and those reacting with both ss and ds DNA. Of these, **high-titre anti-ds DNA antibody is relatively specific for SLE. Another SLE-specific antibody is the anti-sm antibody**.

Clinical Case

A 27-year-old woman was referred to a rheumatology clinic with a history of pain and swelling in the joints of her hand and a rash on her cheeks. Immunofluorescent test for antinuclear antibodies (ANA) showed a homogeneous pattern—anti-ds DNA antibody was positive. LE cells were demonstrated on bone marrow biopsy. A diagnosis of systemic lupus erythematosus (SLE) was made. The patient was treated with low-dose corticosteroids and showed improvement.

- ***Rheumatoid arthritis*** This is a symmetric polyarthritis with muscle wasting and subcutaneous nodules, commonly associated with serositis, myocarditis, vasculitis and other disseminated lesions. It is found more commonly in women. The synovial membranes of the affected joints are swollen and edematous, with dense infiltration of lymphocytes and plasma cells. A striking feature is the presence of a circulating autoantibody called the '**rheumatoid factor**' **(RF)**. This is usually a 19-s IgM, though IgG and IgA RF have also been demonstrated. RF acts as an antibody against the *Fc* fragment of immunoglobulins.
- ***Polyarteritis nodosa*** This is a necrotising angiitis involving medium-sized arteries, ending fatally due to coronary thrombosis, cerebral hemorrhage or gastrointestinal bleeding. Polyarteritis is seen as a component of serum sickness and other toxic complex diseases. Though it has been suggested that polyarteritis nodosa may be an autoimmune disease, the autoantibody responsible has not been identified.
- ***Sjogren's syndrome*** This is a triad of conjunctivitis sicca, dryness of the mouth with or without salivary gland enlargement, and rheumatoid arthritis. The syndrome may occur in association

with other collagen diseases. Antinuclear antibodies and rheumatoid factor commonly occur in sera.

PATHOGENESIS OF AUTOIMMUNE DISEASES

Many diseases are considered to be of autoimmune origin, based on their association with cellular or humoral immune response against self-antigens. The mere presence of autoantibodies during the course of a disease does not prove their causative role. Autoantibody formation may be a result of tissue injury and the antibody may help in promoting immune elimination of the damaged cell or tissue elements.

Antibodies may cause damage by:

- Cytolytic or cytotoxic (Type 2) reactions
- Toxic complex (Type 3) reactions
- Sensitised T lymphocytes (Type 4) reactions

It is likely that humoral and cellular immune responses may act synergistically in the production of some autoimmune diseases. For example, experimental orchitis can be induced only when both types of immune response are operative.

LABORATORY TESTS TO DETECT AUTOIMMUNE DISORDERS

Besides clinical diagnosis based on symptoms, a laboratory confirmation becomes necessary to identify the specific type of autoimmune disorder. The main principle of all diagnostic tests in autoimmune disorders is the capture of autoantibodies from serum using immobilised autoantigens derived from cells. Some of the tests are as follows:

- ***Immunofluorescence*** **Indirect immunofluorescence** is commonly used to detect antibodies against antinuclear antigens, namely ANA including cytoplasmic components. Liver cell lines or HEp2 cells are incubated with the patient's serum. A test is noted as positive if the IgG, IgM or IgA in the patient's serum

attach to the nuclear antigens (in the cell lines).
Anti-human immunoglobulin tagged with a fluorescein dye is used to stain the antigen–antibody complex. This is visualised with a fluorescence microscope.

- ***Enzyme immunoassay (EIA)*** This is widely used for detecting the presence or concentration of individual autoantibodies in biological fluids. It is also used to detect double-stranded DNA and other autoantigens.
- ***Multiplex immunoassay*** This enables the simultaneous detection of different autoantibodies, in which a large number of antigens are immobilised on solid carrier bead-based arrays. This is a relatively new technique.
- ***Anti-CCP (cyclic citrullinated peptide) ELISA*** This test detects human autoantibodies against cyclic citrullinated peptide, which is a circular peptide containing the amino acid citrulline. IgG class of antibodies against this peptide is raised in rheumatoid arthritis (RA). A person positive for rheumatoid factor (RF) and raised anti-CCP has a higher likelihood of progressing to erosive disease. This test also helps to differentiate RA from other types of arthritis. If titres are raised in a healthy individual, the test predicts the development of RA in future.
- ***Rheumatoid factor (RF)*** RA factor can be detected by latex agglutination (LA) and ELISA.

MANAGEMENT OF AUTOIMMUNE DISORDERS

Once initiated, most autoimmune responses tend to be self-perpetuating. Their progress can be arrested by the following measures:

- **Immunosuppressive therapy,** though the degree of response to such therapy varies in different diseases.
- **Vaccination with T cells** specific for a given autoantigen can be administered. These vaccines are synthetic blocking peptides that compete with the autoantigen for binding with the MHC molecules.

- **Monoclonal antibodies** to the self-antigen responsible for the autoimmune reactions can also arrest the progression of autoimmunity.

IMMUNOHEMATOLOGY

ABO System

The ABO system consists of four blood groups and is determined by the presence or absence of two distinct antigens, A and B, on the surface of erythrocytes.

The four groups are also distinguished by the presence or absence of two distinct isoantibodies in the serum. The serum contains the isoantibodies specific for the antigen that is absent in the red cell. The serum of a **group A individual has anti-B antibody, a group B individual has anti-A antibody and group O individuals have both anti-A and anti-B antibodies. In a person of group AB, both anti-A and anti-B antibodies are absent**
(Table 16.1).

In India, the distribution is approximately: O – 40 per cent, A – 22 per cent, B – 33 per cent and AB – 5 per cent.

Anti-A and anti-B isoantibodies appear in the serum of infants by the age of six months and persist thereafter. These are called **'natural' antibodies** because they seem to arise from genetic control without any apparent antigenic stimulation. Natural anti-A and anti-B antibodies are IgM saline-agglutinating antibodies that react optimally between 4°C and 18°C but which are less active at 37°C. Immune isoantibodies may develop following ABO incompatible pregnancy or transfusion.

Table 16.1 Distribution of ABO antigens and antibodies in red cells and serum

Red cells			Serum	
Group	**Antigen present**	**Agglutinated by serum of group**	**Antibody present**	**Agglutinates cells of group**
A	A	B, O	Anti-B	B, AB
B	B	A, O	Anti-A	A, AB
AB	A and B	A, B, O	None	None
O	None	None	Anti-A and anti-B	A, B, AB

H antigen

Red cells of all ABO groups possess a common antigen, the H antigen or H substance, which is a precursor to the formation of A and B antigens. Due to its universal distribution, the H antigen is not ordinarily important in grouping or blood transfusion. Bhende et al. (1952) reported a very rare instance in Mumbai in which A and B antigens as well as H antigens were absent from red cells. This is known as '**Bombay**' or **OH blood**. Such individuals have anti-A, anti-B and anti-H antibodies and their sera are incompatible with all red cells except with those with the same rare blood group.

Rh Blood Group System

The antibody described by Levine and Stetson and identified as the **anti-Rh factor antibody** is found in the red cells of the majority of persons tested. **Rh factor** is an antigen that reacts with rabbit antiserum to Rhesus monkey erythrocytes; hence it is called the 'Rhesus' or Rh factor. Levine and colleagues (1941) proved that Rh sensitisation was the cause of hemolytic disease of the newborn.

Rh typing

For routine purposes, the typing of persons as Rh positive or negative depends on the presence or absence of **antigen D (Rho)** on red cells and hence can be accomplished by testing with anti-D (anti-Rh) serum. The distribution of Rh positives differs in different races. Among people of European descent, about 85 per cent are Rh positive and 15 per cent negative. Among Indians, approximately 93 per cent are Rh positive and 7 per cent are negative.

A variant of D is known as Du. Red cells of the **Du subtype** react with some but not all anti-D sera. Though Du cells may not be agglutinated by anti-D sera, they absorb the antibody on their surface. For the purpose of blood donation, Du cells are considered Rh positive. But when a Du individual requires transfusion, it is advisable to use Rh negative blood because such a person can be immunised by standard Rh positive blood.

There are no natural anti-Rh antibodies in the serum. They arise only as a result of Rh incompatible pregnancy or transfusion.

Medical Implications of Blood Group Antigens

Blood transfusion

The existence of several different blood group antigens makes it almost impossible to obtain perfectly matched blood for transfusion. But in routine transfusion practice, only the ABO and Rh antigens are relevant. The other antigens are too weak to be of importance.

Choice of donor

Safety in blood transfusion requires that the following conditions be satisfied in choosing a donor:

- The recipient's plasma should not contain any antibody that will damage the donor's erythrocytes.
- The donor plasma should not have any antibody that will damage the recipient's red cells.

O group was designated as the '**universal donor**'. The anti-A and anti-B antibodies in the transfused O blood group do not ordinarily cause any damage to the red cells of A or B group recipients. They are rendered ineffective by dilution in the recipient's plasma. However, some O group plasma may contain isoantibodies in high titres (1:200 or above) which result in damage to the recipient's cells. This is known as the **dangerous O group**.

Due to the absence of isoantibodies in plasma, AB group persons were designated '**universal recipients**'. **Rh compatibility is important only when the recipient is Rh negative**. An Rh positive person may safely receive either Rh positive or Rh negative blood. But an Rh negative individual receiving Rh positive blood may form antibodies against the Rh antigen.

Cross-matching

Besides ABO grouping and Rh typing of the donor and recipient, it is invariably necessary to perform cross-matching before transfusion to ensure that the donor's blood is compatible with the recipient's blood.

- ***Coombs cross-match*** The saline slide test does not detect Rh and other minor incompatibilities. The most discriminating method is the Coombs cross-match, where washed donor cells and recipient serum are incubated in a water bath at 37°C for two hours and a direct Coombs test is done. This detects all incompatibilities, including incomplete antibodies.

Hemolytic disease of the newborn

When an Rh negative woman carries an Rh positive fetus, she may be sensitised against the Rh antigen by the passage of fetal red cells into maternal circulation. Minor transplacental leaks may occur any time during pregnancy but it is during delivery that fetal cells enter maternal circulation in large numbers. The mother is usually sensitised only at the first delivery and, consequently, the first child escapes harm. During a subsequent pregnancy, Rh antibodies of the IgG class pass from the mother to the fetus and damage its erythrocytes. This is the pathogenesis of hemolytic disease of the newborn. The clinical features may vary from a mere accentuation of physiological jaundice in the newborn to **erythroblastosis fetalis** or intrauterine death due to **hydrops fetalis**. Hemolytic disease does not affect all the offspring of Rh-incompatible unions.

Detection of Rh antibodies

Most Rh antibodies are of the IgG class, and being 'incomplete antibodies', they do not agglutinate Rh positive cells in saline. A minority are complete (saline-agglutinating) antibodies of the IgM class. These are not relevant in the pathogenesis of hemolytic disease as they do not traverse the placenta.

IgG anti-D antibodies may be detected by the following techniques: (1) using a colloid medium such as 20 per cent bovine serum albumin; (2) using red cells treated with enzymes such as trypsin, pepsin, ficin or bromelin and (3) by the **indirect Coombs test**. The last is the most sensitive method.

Identification of Rh incompatibility

Rh typing forms part of routine antenatal examination. When a woman is Rh negative and her partner Rh positive, fetal complications should be expected. Women with Rh-incompatible pregnancies should be screened for

Rh antibodies by the indirect Coombs test at 32–34 weeks of pregnancy and at monthly intervals thereafter.

When hemolytic disease is diagnosed antepartum, intrauterine transfusion with Rh negative blood may be indicated. Red cells introduced into the fetal peritoneal cavity will find their way into circulation and will survive normally. Premature delivery followed by transfusion may be necessary in some cases. When a baby is born with hemolytic disease, exchange transfusion with Rh negative, ABO-compatible blood is the treatment of choice.

Complications of incompatible blood transfusion

- The red cells may undergo clumping and intravascular hemolysis or they may be coated with antibodies, engulfed by phagocytes, removed from circulation and subjected to extravascular lysis.
- It may be accompanied by symptoms such as shivering, tingling sensation, excruciating headache, constricting precordial discomfort and severe lumbar pain. Hypotension, cold and clammy skin, cyanosis, feeble pulse and other signs of collapse may be seen. Jaundice, hematuria, oliguria and anuria may follow.
- Some transfusion reactions may be due to immunological processes other than blood group incompatibility. Rigor, urticaria and other manifestations often occur when the recipient is hypersensitive to some allergen present in the donor's blood. Serious reactions follow when hemolysed or contaminated blood is transfused.

Key points

- Autoimmune disorders are those in which the immune system produces auto (self) antibodies to endogenous (self) antigens, resulting in tissue injury.
- Usually, the body displays immunological tolerance, wherein the individual's immune system 'tolerates' its own 'self' antigens, and is able to differentiate between 'self' and 'non-self' antigens. Autoimmunity occurs when this tolerance is lost, and the immune system attacks its own tissues. Autoimmune reactions may be mounted by antibody and T cell responses.
- Self-antigens may be intracellular components, receptors, cell membrane components, extracellular components, plasma proteins or hormones; depending on the location of the self-antigen, the disease may be localised to a single organ or may affect multiple sites.
- Autoimmunity may involve antigenic alterations, sequestration, cross-reacting foreign antigens, molecular mimicry or forbidden clones of self-antigens.
- Examples of autoimmune diseases are renal injury (nephritis) associated with systemic lupus erythematosus, orchitis following mumps virus infection, pernicious anemia, thyrotoxicosis (Graves' disease) and Hashimoto's thyroiditis.

- The common treatment is with immunosuppressants but recently, other approaches like monoclonal antibodies and vaccination with specific T cells against self-antigens are also being studied.
- The serum of a particular group carries antibodies specific for the antigen that is absent on the red cell:
    - The serum of group A individuals has antibodies against the antigen that is absent on the red cell, that is, anti-B antibodies; group B individuals have anti-A antibodies; group O individuals have anti-A and anti-B antibodies; whereas group AB individuals possess neither anti-A nor anti-B antibody.
- Blood grouping for ABO groups is done by agglutination tests.
- It is essential to administer blood of the same group as that of the recipient. Before blood transfusions, besides ABO grouping and Rh typing of the donor and recipient, it is necessary to perform cross-matching to ensure that the donor's blood is compatible with the recipient's blood.
- In an emergency, group O blood can be given, since group O RBCs carry no antigens. Group O individuals are termed universal donors. Group AB individuals have neither anti-A nor anti-B antibodies in their serum; they can accept blood of any of the four groups and hence are termed universal recipients.
- If an Rh negative mother conceives a fetus that has Rh positive red blood cells, anti-Rh antibodies from maternal circulation may pass into fetal circulation, damaging fetal red blood cells; erythroblastosis fetalis or even intrauterine death may occur. This usually does not happen with the first pregnancy but only with subsequent pregnancies.

Questions

Short Answers

1. Molecular mimicry
2. Sequestered antigens in autoimmunity
3. Localised (organ-specific) autoimmune disease
4. Hashimoto's disease
5. Systemic lupus erythematosus (SLE)
6. Rheumatoid arthritis
7. Graves' disease
8. Myasthenia gravis
9. Some laboratory tests to diagnose autoimmune disorders
10. Complications of infected blood transfusion
11. Hemolytic disease of the newborn
12. Methods of Rh antibody detection in Rh compatibility testing

CHAPTER 17

Immunology of Transplantation and Tumour Immunity

Chapter navigation

- Transplantation immunity
- Tumour immunity
- Immunomodulators and immunosuppressors

IMMUNOLOGY OF TRANSPLANTATION

Transfer of cells, tissues or organs from one individual to another or from one site to another site in the same individual is known as transplantation. The tissue or organ transplanted is known as the **transplant** or **graft**. The individual from whom the transplant is obtained is known as the **donor** and the individual to whom it is applied, the **recipient**.

Clinical Case

A 38-year-old man with end-stage renal failure due to chronic glomerulonephritis was given a cadaveric kidney transplant. His major blood group was A. The donor kidney was also of blood group A, and was matched for one DR and four of the six ABC antigens. The patient was put on immunosuppressive therapy.

On the third postoperative day, his urea and creatinine levels decreased appreciably. But on the seventh post-operative day, his graft became tender and his creatinine level increased. A clinical diagnosis of acute rejection was confirmed on finding lymphocytic infiltration in the renal cortex biopsy. The patient was started on intravenous corticosteroids and improved 24 hours later. Oral steroids were continued.

Classification of Transplants

Transplants may be classified in several ways based on several parameters:

1. Based on the **organ or tissue transplanted**, they are classified as kidney, heart, skin transplant, and so on.

2. Based on the **anatomical site of origin** of the transplant and the **site of its placement**, grafts are classified as:

- **Orthotopic**—grafts are applied in anatomically 'normal' sites, as in skin grafts.
- **Heterotopic**—grafts are placed in anatomically 'abnormal' sites, e.g., thyroid tissue transplanted into a subcutaneous pocket.

3. Based on whether transplants are from **fresh tissues and organs** or from **stored** ones, or whether they are from **living or dead materials (cadaveric transplants)** (*Clinical case*), they may be classified as follows:

- **Vital grafts**—live grafts such as the kidney or heart, which are expected to survive and function physiologically in the recipient.
- **Structural (static) grafts**—non-living transplants like bone or artery, which merely provide a scaffolding on which new tissue is produced by the recipient.

4. Based on the **genetic (and antigenic) relationship** between the donor and the recipient, they are classified as follows:

- **Autograft—**an organ or tissue taken from an individual and grafted on the same person
- **Isograft—**a graft taken from an individual and introduced into another individual of the same genetic constitution, e.g., grafts made between identical twins or between syngeneic members of highly inbred strains of animals
- **Allograft** (formerly homograft)—grafts between two genetically non-identical members of the same species
- **Xenograft** (formerly heterograft)—grafts between members of different species

Immunological Basis of Allograft Rejection

The immunological basis of graft rejection is evident from the specificity of the response. Accelerated rejection is seen only if the second graft is from the same donor as the first.

First set response

When a skin graft from an animal (such as a rabbit) is applied on a genetically unrelated animal of the same species, the graft appears to be accepted initially. The graft is vascularised and seems morphologically and functionally healthy during the first two or three days. However, by about the fourth day, inflammation becomes evident and the graft is invaded by lymphocytes and macrophages. The blood vessels within the graft are occluded by thrombi, the vascularity diminishes and the graft undergoes ischemic necrosis. With extending necrosis, the graft assumes a scab-like appearance and sloughs off by the tenth day. This sequence of events resulting in the rejection of the allograft is known as the **'first set rejection or reaction'** (Fig. 17.1).

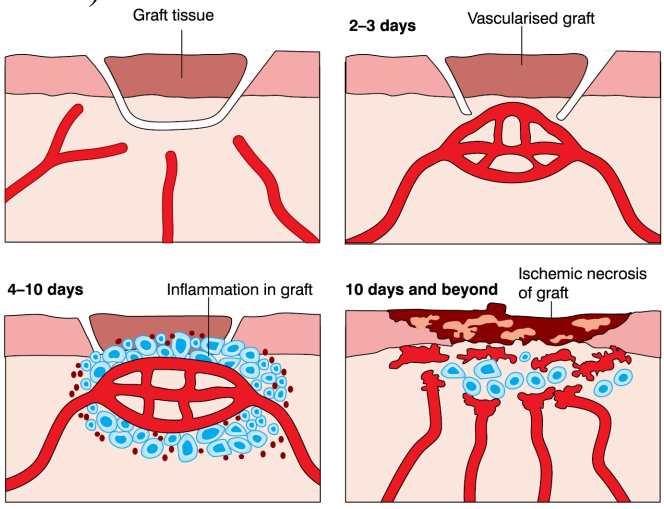


Fig. 17.1 First set rejection: Blood vessels within the graft are occluded by thrombi, the vascularity diminishes and the graft undergoes ischemic necrosis

Second set response

If, in an animal that has rejected a graft by the first set response, another graft from the same donor is applied, it will be rejected in an accelerated fashion, known as the second set response. In this reaction, antibodies are formed more rapidly and abundantly than during primary rejection. Antibodies are believed to participate in the second set response along with cell-mediated immunity (Fig. 17.2).

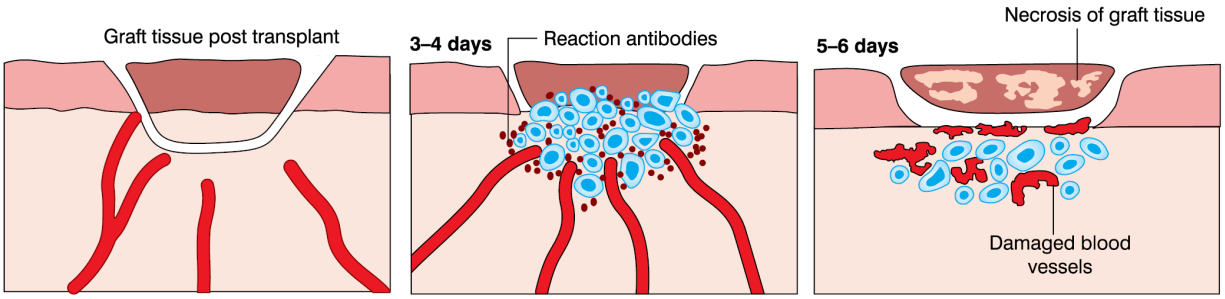


Fig. 17.2 Second set rejection: The reactions are rapid with involvement of antibodies and cell-mediated immunity, resulting in rejection of the graft

Hyperacute rejection (white graft response)

When a graft is applied to an animal possessing the specific donor antibodies in high titres, hyperacute rejection occurs. In such case, pre-formed anti-donor antibodies rapidly bind to antigens on donor tissue and result in the activation of complement or directly recruit host macrophages and neutrophils via their *Fc* region. As a result, microthrombi are formed and vessels are occluded (Fig. 17.3a and b). The graft remains pale and is rejected within minutes to hours without even an attempt at vascularisation. This is known as the white graft response. This type of hyperacute rejection is sometimes seen in human recipients of kidney transplants, who may possess pre-existing antibodies as a result of prior transplantation, transfusion or pregnancy.

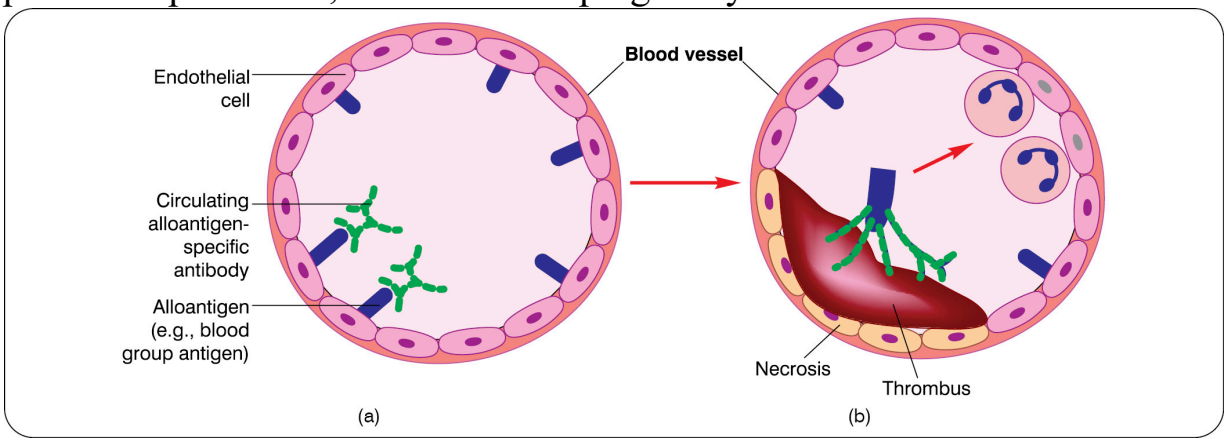


Fig. 17.3 (a) and (b) Vascular occlusion and thrombi formation in hyperacute rejection

Acute rejection

This form occurs within one week to ten days after a transplant has been grafted. This is due to incompatibility of the allograft. The immune response against the graft increases in intensity, with the accumulation of

lymphocytes, plasma cells, macrophages and neutrophils, leading to endothelial damage (Fig. 17.4). There is necrosis and edema, and finally, the graft is rejected.

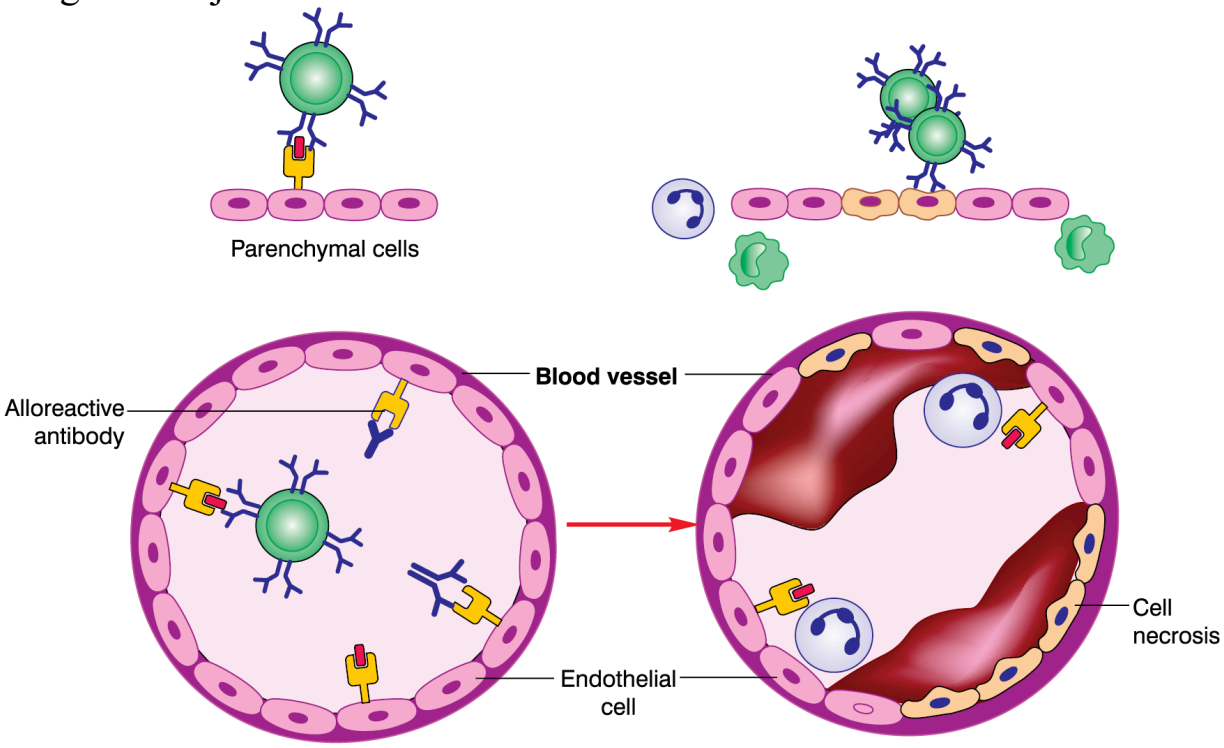


Fig. 17.4 Acute rejection: Accumulation of lymphocytes and plasma cells, leading to endothelial damage

Chronic rejection

This is a late-onset rejection occurring after 60 days or more, often superimposed by acute changes (Fig. 17.5). In kidney transplantation, there is mesangial cell proliferative glomerulonephritis and interstitial fibrosis. The patients respond poorly to corticosteroids.

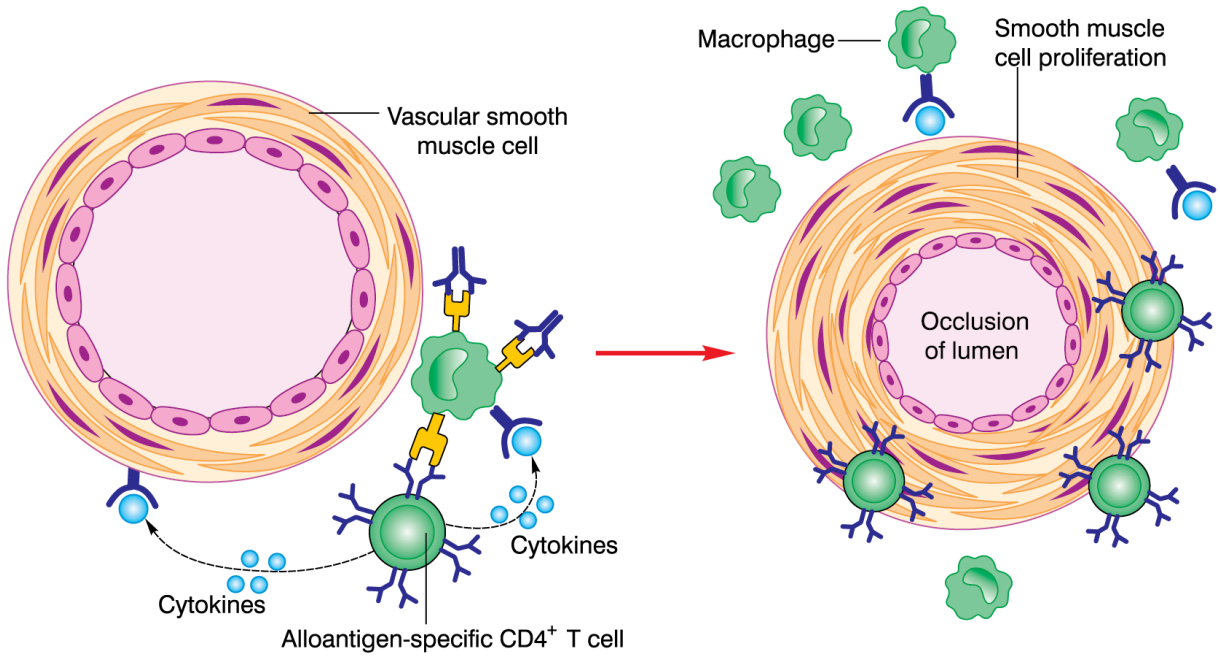


Fig. 17.5 Chronic rejection: Late-onset rejection initiated by immune mechanism with infiltration of granulocytes, and onset of fibrosis

Transplantation immunity is predominantly cell-mediated. The first set response is brought about almost exclusively by T lymphocytes. Humoral antibodies are also produced during allograft rejection. First response is a sensitisation phase, in which antigen-reactive lymphocytes of the recipient proliferate in response to allo-antigen on the graft. This is followed by an effector stage in which immune destruction of the graft takes place (Fig. 17.6).

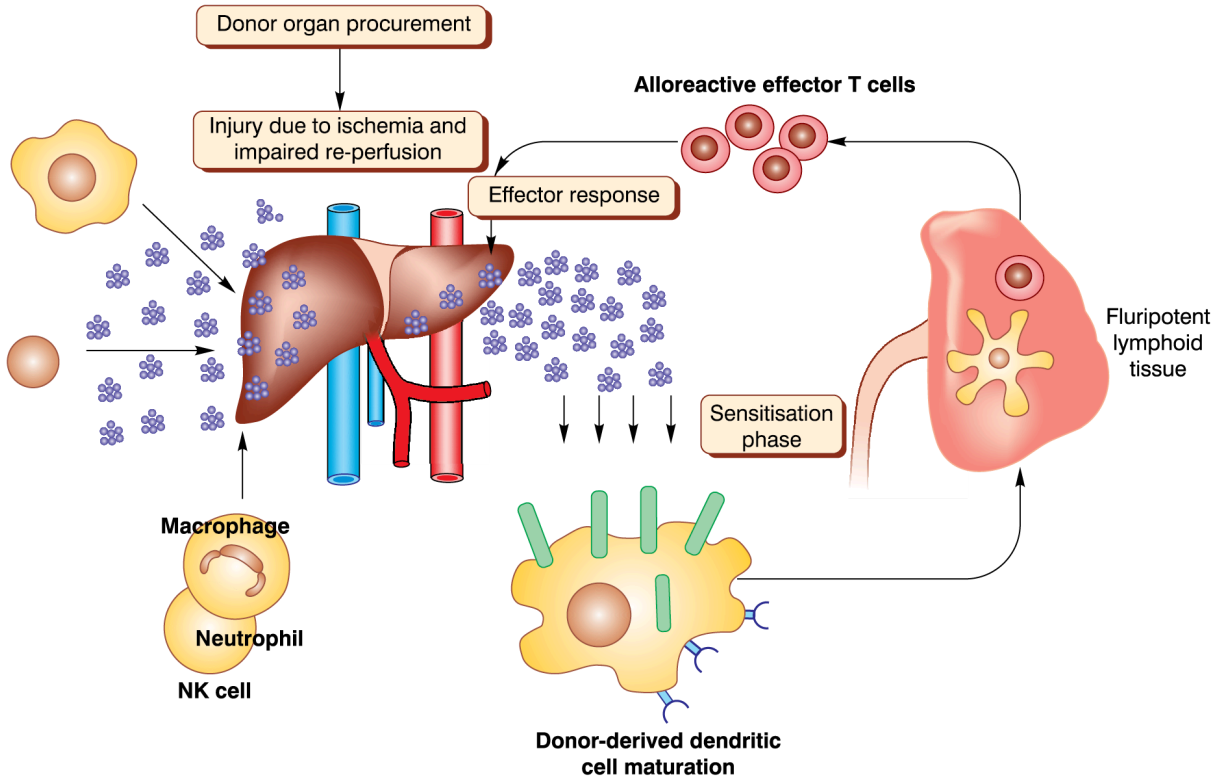


Fig. 17.6 Mechanism of graft rejection initiated by sensitisation of the immune cells followed by effector mechanism leading to rejection

Adoptive Immunisation

An allograft will be accepted if the animal is rendered immunologically tolerant. The method of transferring immunity by means of lymphoid cells is known as adoptive immunisation.

Immunological enhancement

Humoral antibodies may sometimes oppose cell-mediated immunity by inhibiting graft rejection. This phenomenon, called immunological enhancement, was originally described by Kaliss in tumour transplants. If the recipient is pre-treated with one or more injections of killed donor tissue and the transplant applied subsequently, it survives much longer than in control animals. The enhancing effect can be passively transferred to normal animals by an injection of serum from immunised animals, showing that the effect is due to humoral antibodies.

The antibodies may bring about the enhancing effect in various ways.

- They may combine with the antigens released from the graft so that they are unable to initiate an immune response (**afferent inhibition**).
- The antibodies may combine with the lymphoid cells of appropriate specificity and, by a negative feedback influence, render them incapable of responding to the antigens of the graft (**central inhibition**).
- They may also coat the surface of cells in the graft so that sensitised lymphocytes are kept out of contact with them (**efferent inhibition**).

Allograft immunity is a generalised response directed against all the antigens of the donor. A recipient sensitised by a skin graft will reject, by the second set response, not only another skin graft but also any other organ or tissue graft from the same donor (Fig. 17.7).

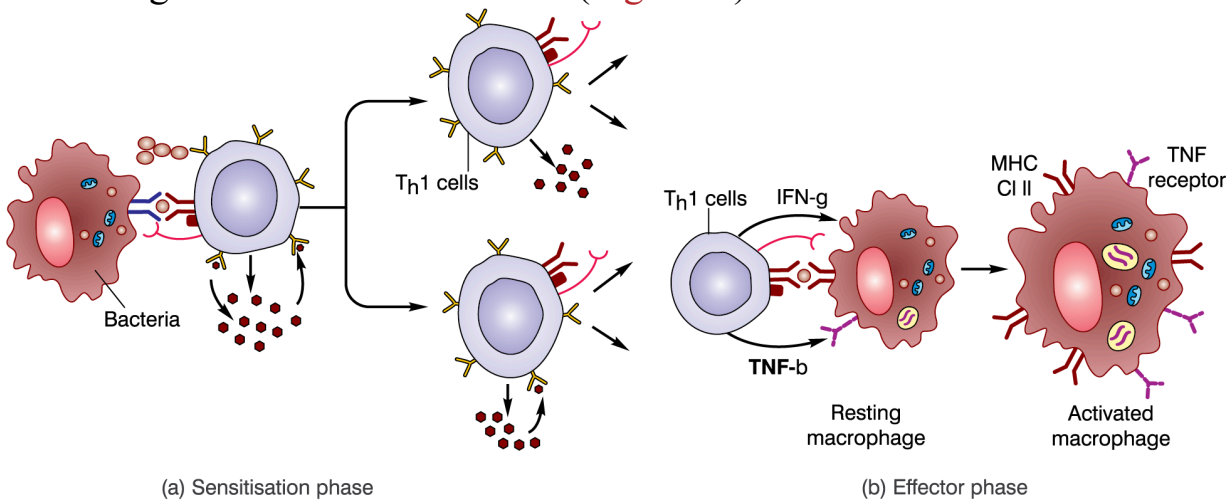


Fig. 17.7 (a) and (b) Allograft reaction

Histocompatibility Antigens

Immune response against transplants depends on the presence of antigens in the grafted tissue, which are absent in the recipient and hence recognised as foreign. Therefore, if the recipient possesses all the antigens present in the graft, there will be no immune response, and consequently no graft rejection, even when the donor and recipient are not syngeneic. The first generation (F_1) hybrids between two inbred strains possess antigens

representative of both the parent strains and will, therefore, accept grafts from either of the parental strains. If the two parental strains have genotypes AA and BB, respectively, the F_1 hybrid will be of genotype AB. It can, therefore, accept tissues with genotype AA as well as BB, as it possesses both alleles. Transplantation in the reverse direction (from F_1 to parent) will not succeed as strain AA will react against antigen B and strain BB against antigen A.

Eichwald–Silmser effect

While transplants between members of a highly inbred strain of animals are successful, an exception is seen when the donor is a male and the recipient a female. Such grafts are rejected as the grafted male tissue (XY) will have antigens determined by the Y chromosome which will be absent in the female (XX) recipient. Grafts from the female to the male will succeed. This unilateral **sex-linked histoincompatibility** is known as the Eichwald–Silmser effect.

Antigens that participate in graft rejection are called **transplantation or histocompatibility antigens**. The term 'major histocompatibility system' refers to a system of cell antigens that exert a decisive influence on the fate of allografts. The major histocompatibility system in human beings is the human leucocyte antigen (HLA) system.

Histocompatibility Testing

- ***Blood grouping*** ABO blood group antigen compatibility is important in transplantation.
- ***HLA compatibility*** Next to ABO blood group compatibility, the most important factor in allograft survival is HLA compatibility. This is tested by HLA typing and tissue matching. HLA typing identifies the HLA antigens expressed on the surface of leucocytes.

Methods of HLA typing

- ***Microcytotoxicity test*** Lymphocyte suspensions are added to microwells of tissue typing trays predispensed with a panel of HLA typing sera, each containing alloantibodies to a specific

HLA antigen, and incubated with complement. Cells carrying antigens corresponding to the HLA antiserum are killed by complement-mediated membrane damage. These can be detected by the addition of eosin or trypan blue which stains only dead cells. The lymphocyte is presumed to have HLA antigens corresponding to the specificities of all the antisera that have caused cell death, as indicated by the staining. Antisera for HLA typing were originally obtained from multigravidae, placental fluid and from multiple blood transfusion recipients, who have antibodies against mismatched paternal or donor HLA antigens. These are now being replaced by monoclonal antibodies.

- ***Molecular methods*** More discriminating molecular methods have been developed for tissue typing. These include **restriction fragment length polymorphism (RFLP) with Southern blotting** and **polymerase chain reaction (PCR) amplification** using sequence-specific primers.
- ***Tissue matching*** Once a set of HLA-compatible donors is available (commonly, siblings of the patient), the best among them can be chosen by tissue matching. This is done by the mixed lymphocyte reaction or culture (MLR and MLC). It depends on the fact that T lymphocytes in culture, when exposed to HLA-incompatible antigens, will undergo blast transformation, the intensity of the reaction being a measure of the antigenic disparity between the donor and recipient lymphocytes. This is a one-way test in which donor lymphocytes are killed and only recipient lymphocytes are permitted to be transformed in response to the incompatible antigens on the donor cells.

Immunosuppression

As allograft rejection is an immunological process and immunosuppression will inhibit it. This can be achieved in experimental animals by neonatal thymectomy, chronic lymphatic drainage or the administration of ALS—procedures that will inhibit cell-mediated immunity.

Clinical transplantation employs a combination of immunosuppressive drugs, including steroids, azathioprine and the fungal metabolite

cyclosporin A, which is currently the most effective agent.

Privileged sites

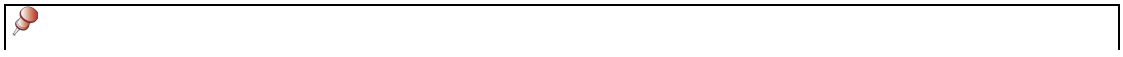
There appear to be certain privileged sites where allografts are permitted to survive, safe from immunological attack. The fetus can be considered an intrauterine allograft as it contains antigens foreign to the mother. Why the fetus is exempt from rejection is not clear, though many explanations have been offered. The placenta acts as an immunological barrier by generating a locally immunosuppressive hormone. Major histocompatibility complex (MHC) antigens are present only in low density on trophoblastic cells and the cell membranes are relatively resistant to attack by T or K cells. Antigen shedding by the fetus blocks the aggressive T cells or antibodies by an enhancement effect. An incomplete mucopolysaccharide barrier rich in sialic acid surrounds the trophoblastic cells, protecting them from cytotoxic lymphocytes. The high concentration of alpha-fetoprotein in fetal blood may also be a factor, as it has immunosuppressive properties which may protect the fetus against immunological damage from any maternal leucocytes entering fetal circulation.

Any site that is impenetrable to immunocompetent cells (e.g., cartilage) is an immunologically privileged site. Areas in which a lymphatic drainage system is absent (e.g., brain or hamster cheek pouch) or ineffective (e.g., testes) can accept allografts without rejection. Lack of vascularity at the site also prevents graft rejection. This is the reason for the success of corneal transplants.

Graft-versus-host reaction

In this condition, the graft mounts an immune response against the antigens of the host. This is known as the graft-versus-host (GVH) reaction. This occurs when the following conditions are present:

- The graft contains immunocompetent T cells.
- The recipient possesses transplantation antigens that are absent in the graft.
- The recipient must be incapable of mounting an effective response to eliminate the graft immunocompetent cells.



Examples of situations leading to the GVH reaction

- Allograft in a recipient in whom specific immunological tolerance has been induced
- Adult lymphocytes injected into an immunologically deficient recipient in whom the deficiency is due to immaturity (newborn) or immunosuppression
- F_1 hybrid receiving a transplant from any one parental strain

The main clinical features of the GVH reaction in animals are retardation of growth, emaciation, diarrhea, hepatosplenomegaly, lymphoid atrophy and anemia, terminating fatally. The syndrome has been called **runt disease**.

Clinical Transplantation

Organ/tissue transplantation is performed for various illnesses in India and across the world. Although the frequency of cornea, kidney, skin, heart, lung, liver and bone marrow transplants is increasing, the availability of organ donors is a challenge that might be solved in the future by innovations in transplantation and stem cell technology.

IMMUNOLOGY OF MALIGNANCY

When a cell undergoes malignant transformation, it acquires new surface antigens. It may also lose some normal antigens. This makes a tumour antigenically different from the normal tissues of the host. A tumour can, therefore, be considered an allograft and be expected to induce an immune response.

Clinical Evidence of Immune Response in Malignancy

Several clinical observations indicate the presence of an immune response that prevents, arrests and occasionally cures malignancies.

- ***Spontaneous regression*** Instances of spontaneous regression of established tumours have been reported, especially with neuroblastoma and malignant melanoma. Based on the role played by the immune response in recovery from infections, it is

believed that recovery from malignancy may also represent an immune process.

- ***Chemotherapy cures*** Dramatic cure sometimes follows chemotherapy of choriocarcinoma and Burkitt's lymphoma. Even a single dose of a cytotoxic drug may, on occasion, result in complete cure. Again, in some types of tumours, such as hypernephroma with pulmonary metastases, the removal of the primary tumour often leads to regression of the metastases. These observations suggest that once a large mass of tumour has been removed, mopping up operations can be effected by the immune process. The immune response appears to be effective only when the tumour is below a 'critical mass'.
- ***Overcome defence mechanisms*** There is a higher prevalence of certain types of cancers observed unexpectedly at autopsy than their clinical incidence would suggest. This indicates that the immune system is able to deal with malignant cells as they arise and that only some of them overcome the defence mechanisms and develop into clinical cancer.
- ***Cellular response*** Histological evidence of immune response against malignancy is provided by the presence of lymphocytes, plasma cells and macrophages infiltrating tumours. The cellular response resembles that seen in the allograft reaction. Tumours showing such cellular infiltration have a better prognosis than those that do not.
- ***Immunodeficiency states*** If the immune system plays a natural role in preventing tumour development, a high incidence of malignancy should be expected in immune deficiency states. This is indeed so. Increased incidence of cancer, particularly lymphoreticular malignancies, is found in congenital immunodeficiency states, in AIDS and in patients undergoing chronic immunosuppressive therapy.

Tumour Antigens

- ***Tumour-specific antigens*** These antigens are present in malignant cells but absent in the corresponding normal cells of the host. They induce an immune response when the tumour is transplanted in syngeneic animals. Such antigens, which induce rejection of tumour transplants in immunised hosts, are termed **tumour-specific transplantation antigens** (TSTA) or **tumour-associated transplantation antigens** (TATA). In chemically induced tumours, the TSTA is tumour-specific. Different tumours possess different TSTA, even though they are induced by the same carcinogen. In contrast, the TSTA of virus-induced tumours is virus-specific in that all tumours produced by one virus will possess the same antigen, even if the tumours occur in different animal strains or species.
- ***Tumour-associated antigens*** This type of antigen is found in some tumours and may also be present in a few normal cells:
    - **Oncofetal antigens** are **fetal antigens** which are found in embryonic and malignant cells but not in normal adult cells. The best known examples are alpha-fetoprotein in hepatomas. Their synthesis represents a de-differentiation of malignant cells into more primitive forms.
    - The **carcinoembryonic antigen** is a glycoprotein that can be detected in the serum of many patients with carcinoma of the colon, particularly in the presence of metastases. However, it also appears in some other conditions such as alcoholic cirrhosis. For this reason, its diagnostic value is limited. Alpha-fetoprotein is an alpha-globulin secreted by normal embryonic hepatocytes. Its serum level drops sharply after birth and is hardly detectable in adults. High levels are present in hepatic carcinoma, in which condition, it is of diagnostic value.
    - **Differentiation antigens** include those such as the prostate-specific antigen (PSA), whose level is higher in patients of prostate cancer and has been used as a diagnostic indicator. Similarly, CA125

(cancer/carbohydrate antigen 125) is widely used as a diagnostic and prognostic marker for ovarian cancer.

Immune Response in Malignancy

Both humoral and cellular responses can be demonstrated in malignancy. Anti-TSTA antibodies can be demonstrated by indirect membrane immunofluorescence. Delayed hypersensitivity to tumour antigens can be detected by skin testing with tumour cell extracts. Cell-mediated immunity (CMI) can be demonstrated by the stimulation of DNA synthesis and lymphokine production by the patient's leucocytes on exposure to the tumour antigens. The lymphocytes from the patient are cytotoxic to the cultured tumour cells.

CMI is believed to be the mechanism of host defence against malignancy. The humoral response may not be relevant, or may even be detrimental, due to its facilitation of tumour growth by the process of enhancement.

Immunological Surveillance

The primary function of cell-mediated immunity is to '**seek and destroy**' malignant cells that arise by somatic mutation. Such malignant mutations are believed to occur frequently and would develop into tumours but for the constant vigilance of the immune system. Inefficiency of the surveillance mechanism, either as a result of ageing or in congenital or acquired immunodeficiencies, leads to increased incidence of cancer. (The concept of immunological surveillance was developed by **Lewis Thomas** in the 1950s.)

Mechanisms

If immunological surveillance is effective, cancer should not occur. The development of tumours represents a lapse in surveillance. The mechanisms of such lapses are not clear but several possibilities have been suggested:

- Due to the very fast **rate of proliferation** of malignant cells, they may be able to 'sneak through' before the development of an

effective immune response and, once they reach a certain mass, may be beyond the power of immunological attack.

- **Circulating tumour antigens** may act as a **'smokescreen'**, coating the lymphoid cells and preventing them from acting on the tumour cells. The tumour antigens on malignant cells may be inaccessible to sensitised cells, being covered by some antigenically neutral substance. Humoral antibodies may cause immunological enhancement. 'Blocking' activity has been demonstrated in humoral factors. This may be due to the circulating antigen, antibody or antigen–antibody complexes.

Some tumours may be of **low immunogenicity** or may form cytokines, like the transforming growth factor (TGF), which suppresses CMI.

Tumour cells frequently express **low levels of class I MHC molecules** and hence may not be recognised by $CD8^+$ CTLs for destruction. Further, poor immunogenicity and co-stimulatory signals may lead to clonal anergy.

Immunotherapy of Cancer

Different approaches have been used for the immunotherapy of cancer—passive, active and adoptive immunotherapy, specific and non-specific.

Passive immunotherapy

A special type of serotherapy has been found to be beneficial in experimental tumours. Appropriate antisera that possess 'de-blocking' activity in vitro have been found to cause regression of tumours, apparently by neutralising the circulating tumour antigens and permitting the sensitised lymphocytes to act on tumour cells. Monoclonal antibodies to tumour antigens may play a role as carriers in transporting cytotoxic or radioactive drugs specifically to the tumour cells.

Specific active immunotherapy

This method has been modified recently by using purified tumour cell membrane antigens and tumour cells treated with neuraminidase to increase their immunogenic potential.

Non-specific active immunotherapy

This method uses BCG and non-living *Corynebacterium parvum*. Mathe,

the leading proponent of cancer immunotherapy, reported very good results in acute leukemia, following combined treatment with BCG and allogeneic or autochthonous leukemia blast cells. Intralesional BCG in malignant melanoma has been reported to induce complete remission in a high percentage of patients. It has also been used against the intradermal recurrence of breast cancer following mastectomy. Dinitrochlorobenzene has been used in the treatment of squamous and basal cell carcinoma of the skin. Glucan, a pyran copolymer derived from microorganisms, and levamisole, originally introduced as an anthelmintic, have been used to stimulate CMI and macrophage function. Interferons have been employed in the treatment of leukemias.

Specific adoptive immunotherapy

This has been attempted with lymphocytes, transfer factor and 'immune RNA'. The donors have been persons cured of their neoplasms or specifically immunised against the patient's tumour. Lymphokine-activated killer (LAK) cells obtained by the treatment of the natural killer cells with interleukin-2 have been found useful in the treatment of certain malignancies, such as renal carcinomas.

Immunotherapy is ineffective in the presence of a large mass of tumour cells. Its role appears to be important in getting rid of the residual malignant cells after the gross tumour has been removed. The best results in the treatment of cancer apparently follow an integrated approach to therapy, combining surgery, radiotherapy, chemotherapy and immunotherapy.

Immunomodulation

Immunomodulators have the ability to stimulate natural and acquired defence mechanisms, such as cytokines, which enable the immune system to help itself. The natural immunomodulators act to strengthen weak immune systems and to moderate immune systems that are overactive. Plant sterols and sterolins are natural immunomodulators found in some raw fruits and vegetables. Immunomodulators may be either immunosuppressants or immunostimulants.

Immunosuppressive agents are used to inhibit the immune response in transplant rejection and autoimmunity. **Immunostimulants** are used in immunodeficiency conditions, cancers and in some infections (Table 17.1).

Table 17.1 Immunosuppressive and immunostimulating agents and their therapeutic uses

Agents		Therapeutic uses
Immunosuppressive agents		
Cytotoxic agents	Azathioprine, methotrexate, cyclophosphamide	Organ transplantation Autoimmune disorders
Antimetabolites, alkylating agents		
Cytokine inhibitors	Anticytokine-antibodies, TNFα inhibitors, IL-1 inhibitors, and IL-2 inhibitors	
Antibodies against specific immune cell molecules [e.g., Antithymocyte globulin (ATG)]	Polyclonal antibodies, monoclonal	
Immunostimulants		
Recombinant cytokines	Interferons (α, β, γ)	Malignant melanoma, follicular lymphoma, AIDS-related Kaposi's sarcoma
	Interleukins	Metastatic renal cell carcinoma and melanoma
	Colony-stimulating factors (CSF)	Leucopenia, ganciclovir-induced neutropenia
Bacillus Calmette-Guerin (BCG)	Produces granulation tissue at the site of injection	Used for prophylaxis of primary and recurrent-stage papillary tumours after transurethral resection
Levamisole	Used as an adjuvant therapy with 5-fluorouracil	After surgical resection in patients with Duke's stage C colon cancer and agranulocytosis
Cancer vaccines	Several immunomodulants Still in research or phases of clinical trial	

Key points

- Transplants may be classified based on the organs or tissue transplanted, the anatomical site of origin of the transplant and its placement. Based on the genetic relationships,

transplants can be classified as:
    - Autograft (taken from an individual and grafted on himself)
    - Isograft (between individuals of the same genetic constitution, like identical twins)
    - Allograft (between two genetically non-identical members of the same species)
    - Xenograft (between members of different species)
- First set response refers to the sequence of events resulting in the rejection of an allograft.
- In second set response, there is accelerated rejection of another graft from the same donor.
- An allograft is accepted if the host is rendered immunologically tolerant. Adoptive immunisation is the process of transferring immunity by means of lymphoid cells.
- Transplantation immunity is predominantly cell-mediated. The first set response is brought about almost exclusively by T lymphocytes, while in the second set response, antibodies participate along with cell-mediated immunity.
- Immunological enhancement is a phenomenon wherein pre-existing antibodies prevent CMI from causing graft rejection.
- Histocompatibility (transplantation) antigens participate in graft rejection. Major histocompatibility system refers to a system of cell antigens that exerts a decisive influence on the fate of allografts. The human leucocyte antigen (HLA) system is the major histocompatibility system in humans.
- The three classes of HLA antigens are I, II and III. HLA class I antigens (A, B, C) are the principal antigens involved in graft rejection and cell-mediated cytolysis.
- The most important factors favouring allograft survival are the ABO blood group antigens and the HLA antigens. HLA compatibility is tested for by HLA typing and tissue matching.
- Any site that is impenetrable to immunocompetent cells (cartilage) is an immunologically privileged site. Graft rejection is prevented in areas where there is no lymphatic drainage (brain) or where there is lack of vascularity (cornea).
- In the graft-versus-host (GVH) reaction, the graft mounts an immune response against the antigens of the host.
- When a cell undergoes malignant transformation, it acquires new surface antigens and may also lose some antigens, making it antigenically different from the normal tissues of the host.
- Tumour-specific antigens are present in malignant cells but absent in the corresponding normal cells of the host such as tumour-specific transplantation antigens (TSTA).
- Tumour-associated antigens are found in some tumours and may also be present in a few normal cells such as fetal antigens, which are found in embryonic and malignant cells but not in normal adult cells. Some examples are alpha-fetoprotein in hepatomas, carcinoembryonic antigen in colonic cancers and CA125 in ovarian cancers.
- Both humoral and cellular responses can be demonstrated in malignancy; cell-mediated immunity is the probable mechanism of host defence against malignancy.
- Lapses in immune surveillance allow tumours to emerge and may result in proliferation of malignant cells, inaccessibility of tumour antigens on malignant cells to immunocompetent

cells and lowered cell-mediated and humoral immune responses.

- Different approaches have been attempted in the immunotherapy of cancer:
    - Passive immunotherapy, using antisera against TSTA, was found to be of no use.
    - Monoclonal antibodies to tumour antigens have been used as carriers to transport cytotoxic or radioactive drugs specifically to tumour cells.
    - Non-specific active immunotherapy uses BCG and non-living *Corynebacterium parvum.*
    - Specific adoptive immunotherapy has been attempted with lymphocytes, transfer factor and 'immune RNA'. LAK cells have been found useful in the treatment of certain malignancies. Immunotherapy is ineffective in the presence of a large mass of tumour cells.

Questions

Short Answers

1. Features of an allograft reaction
2. Graft-versus-host reaction
3. Histocompatibility antigens
4. Methods of HLA typing
5. Tumour antigens
6. Immune surveillance
7. Immunotherapy in cancer

Part III: Bacteriology

Part III: Bacteriology

Chapter 18

Normal Microbial Flora of the Human Body

Chapter navigation

- Normal flora
- Altered flora
- Normal flora and immunity
- Therapeutic role of normal gut flora

Every human body surface that is exposed to or has access to the environment (e.g., skin, eyes or respiratory and digestive tracts) has a microbiome (microbial population found normally in the body part). A wide array of microorganisms colonise both human as well as animal bodies. These are known as the **normal microbial flora** of the body. They are also known as the **resident flora or commensals.** Normal flora vary in number and type in various sites. Internal organs like the central nervous system, blood, lower bronchi and alveoli, spleen, liver and kidneys do not harbour microorganisms.

Bacteria and yeasts are common constituents of normal flora (Table 18.1). Viruses and protozoa may be present in asymptomatic individuals but are not considered as commensals.

Transient flora refers to organisms that are present at some point of time on the host. They may be potentially pathogenic, in which case, the individual (host) could be a source of infection. An individual who harbours such organisms is known as a **carrier.** Individuals who harbour these organisms for long periods of time are known as **chronic carriers.**

Table 18.1 Normal microbial flora of the body

Site of the body	Normal microbial flora

Skin and hair	Diphtheroids (including propionibacteria) Staphylococci (coagulase-positive and -negative) Micrococci Gram-positive, aerobic, spore-bearing bacilli, *S. viridans* *Candida* species
Conjunctiva	Diphtheroids (*Corynebacterium xerosis*), *Moraxella* species, staphylococci and non-hemolytic streptococci
Nose, nasopharynx and sinuses	Corynebacteria, staphylococci and streptococci *Haemophilus* species and *Moraxella lacunata*
Mouth and upper respiratory tract	Anaerobic micrococci, microaerophilic and anaerobic streptococci, fusiform bacilli, *Corynebacterium* species, *Actinomyces*, *Leptothrix*, *Neisseria* and *Bacteroides*
Gastrointestinal tract	Enterobacteriaceae, enterococci, clostridia *Bacteroides*, several other anaerobic and aerobic bacteria and yeasts
Genitourinary tract	*G. vaginalis*, alpha-hemolytic streptococci and *Bacteroides* species *C. trachomatis* and *Ureaplasma urealyticum, Listeria*, anaerobic streptococci and yeasts

ROLE OF NORMAL MICROBIAL FLORA

Some host–bacterial associations have developed into beneficial relationships, creating an environment of symbiosis. The benefits of such an association include:

- It provides the host with some essential nutrients (e.g., microbial flora of the intestinal tract synthesise vitamin K and several B group of vitamins).
- It aids the metabolism of indigestible compounds.
- It provides defence against colonisation by opportunistic pathogens by providing a local barrier against them.
- It aids the host's immunity—the immune system being dependent on interactions with the human microbiome, as suggested by recent studies.
- It helps in the prevention of inflammatory disease during colonisation as shown by some symbiotic bacterial species; the production of metabolic waste products such as acetic, butyric

and lactic acids by strict anaerobes inhibits the growth of pathogenic bacteria in the large bowel.

Some of the normal microbial flora can act as **opportunistic pathogens** and cause disease in immunocompromised individuals. Normal microbial flora often contaminate clinical samples from sterile sites (due to improper collection), causing confusion in laboratory diagnosis.

The **human skin** is colonised with organisms present in the environment (Table 18.1). The natural flora of the body depends on the area of the body, the clothing one wears, one's occupation and environment. Transient microbial flora tend to colonise the skin more frequently. Often, the skin of the face, neck, hands and buttocks carries pathogenic hemolytic streptococci and staphylococci, and candida. Antibiotic-resistant staphylococci are seen in individuals working in hospitals.

The **conjunctiva** is relatively free from pathogenic organisms due to the flushing action of tears; however, some bacteria are found as commensals.

The **nasopharynx of the infant** is sterile at birth but within 2–3 days, it acquires the common commensal flora and the pathogenic flora carried by the mother and the attendants. The nasopharynx can be considered the natural habitat of some common potentially pathogenic bacteria which cause infections of the nose, throat, bronchi and lungs.

The **mouth** contains several organisms. The gum pockets between the teeth, and the crypts of the tonsils have a wide spectrum of anaerobic flora —all found to varying extents. Among fungi, candida is known to colonise the oral cavity.

The **mouth of the infant** is not sterile at birth. It generally contains the same types of organisms in about the same relative numbers as those present in the mother's vagina, that is, a mixture of micrococci, streptococci, coliform bacilli and Doderlein's bacilli. These organisms diminish in number during the first 2–5 days after birth and are replaced by the types of bacteria present in the mouths of the mother and the nurse.

Within 12 hours of birth, alpha-hemolytic streptococci are found in the upper respiratory tract and become the dominant organisms of the oropharynx and remain so
for life.

Few bacteria are found in the normal bronchi. Smaller bronchi and alveoli

are normally sterile.

Normal flora of the **gut** is the largest symbiotic system in the host. It has been shown to play important roles in maintaining intestinal homeostasis and regulating the maturation of the mucosal immune system. Pathogenic bacteria, on the other hand, cause dysfunction of the immune system, resulting in disease. The majority of the gut flora are anaerobes (*Bacteroides*, clostridia, *Bifidobacterium* and *Fusobacterium*) while the coliform bacteria make up the rest (Fig. 18.1).

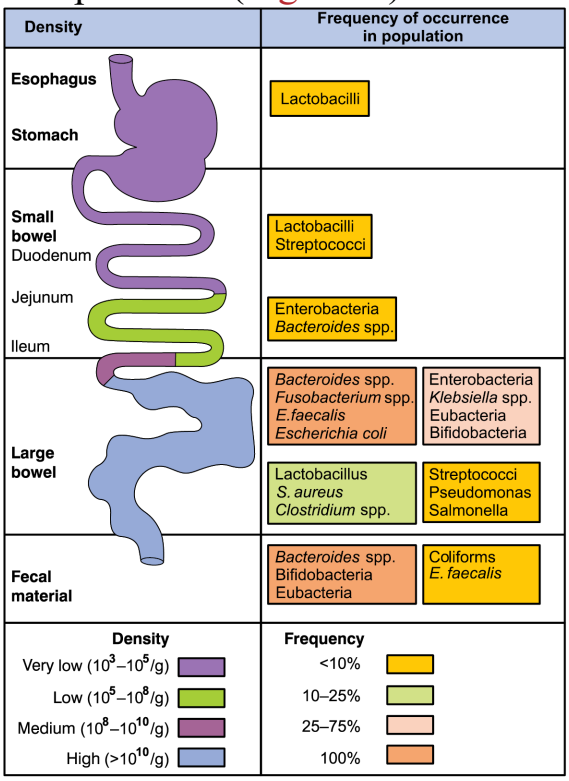


Fig. 18.1 Longitudinal distribution, frequency of occurrence and densities of the bacteria making up the normal flora of the human gastrointestinal tract

The nature of the flora in the vagina depends on the pH of its secretions and its enzyme content. At puberty, the pH becomes acidic due to the metabolic activity of Doderlein's bacilli, *E. coli* and yeasts. This change probably helps prevent colonisation by possibly harmful microorganisms. During pregnancy, there is an increase in *Staphylococcus epidermidis*, Doderlein's bacilli and yeasts. After menopause, the flora resembles that found before puberty. The normal vaginal flora often includes *G. vaginalis,* alpha-hemolytic streptococci, *Bacteroides* species and candida.

Bacteria in the blood and tissues

The commensals from the normal flora of the mouth, nasopharynx and intestinal tract may get into the blood and tissues. They are usually quickly eliminated by the normal defence mechanisms of the body.

PROBIOTICS

Live microorganisms usually found in the normal human body, when administered in adequate amounts, confer health benefits on the host. These microorganisms are referred to as probiotics. The inhibition of other microbes, effects on the mucosal barrier function and interaction with antigen-presenting dendritic cells are some of the functions of probiotics. They have been successfully used in antibiotic-associated diarrhea. The two most common probiotics are:

- *Bifidobacterium*
- *Lactobacillus*

Probiotics can have multiple interactions with the host, including competitive inhibition of pathogenic bacteria.

NORMAL FLORA AND THE IMMUNE SYSTEM

Normal flora induces an immune response at lower levels than pathogenic bacteria. They provide minimum stimulation for the human body so that the immune system remains tuned. However, the exact mechanism and the role of the immune system regulation of the normal flora is not known.

Implication of Alteration of Normal Bacterial Flora

As has been mentioned earlier, there exists a delicate balance between the human body and the microbial flora that live symbiotically with the host or as commensals. Any factor affecting this balance results in adverse effects. Pseudomembranous colitis is one of the classic examples of such an effect.

Some of the major factors responsible for altering the balance and causing infection are:

i) Prolonged antibiotic therapy, immunosuppressive agents and immunodeficiency diseases

ii) Alteration of pH (as in the genitourinary tract in the elderly and during pregnancy)
iii) Increased virulence of the commensals, production of toxins and change in microbial flora

Pseudomembranous colitis

Indiscriminate administration of antibacterial agents may completely eliminate the resident microorganisms, permitting exogenous and endogenous pathogens to gain the upper hand and cause disease. In particular, when patients are treated with broad-spectrum antibiotics administered orally, diarrhea may be a side effect of overgrowth of yeast or staphylococci. When certain drugs, especially clindamycin, are given, the anaerobic gram-positive bacillus, Clostridium difficile, multiplies due to the suppression of other members of the flora; this may result in a serious, life-threatening condition known as pseudomembranous colitis. Hence, wherever possible, narrow-spectrum antibiotics should be used at the correct dosage and for the correct duration of time to prevent the suppression of gut flora.

THERAPEUTIC USE OF GUT FLORA

Trials on the use of gut flora or fecal flora in treating chronic gastrointestinal ailments have been tried and found to give promising results.

Fecal Microbiota Transplantation

Gut bacteria from a healthy donor are introduced into a patient by an infusion of a fecal sample via the nasogastric tube, nasoduodenal tube, rectal enema or the biopsy channel of a colonoscope. These bacteria colonise the gut. Fecal microbiota transplantation is now used in the treatment of conditions like the irritable bowel syndrome.

Key points

- The normal microbial flora are more or less constant for each species and are broadly divided into residents and transients.
- The flora can become pathogenic when host defences falter; they prevent or interfere with colonisation/invasion of the body by pathogens and raise the overall immune status of the host against pathogens.
- Normal flora cause confusion in diagnosis due to their ubiquitous presence in the body and their resemblance to some pathogens.

Questions

Essays

1. Give an account of normal flora in health and disease.

Part III: Bacteriology

Chapter 19

Antimicrobial Agents

Chapter navigation

- Antimicrobial chemotherapy and antibiotic resistance
- Antibiotic policy

Antibiotics act by selective toxicity, i.e., they selectively inhibit the growth of microorganisms without compromising the host cell. There are four major sites on the bacterial cell that antibiotics act on (Fig. 19.1).

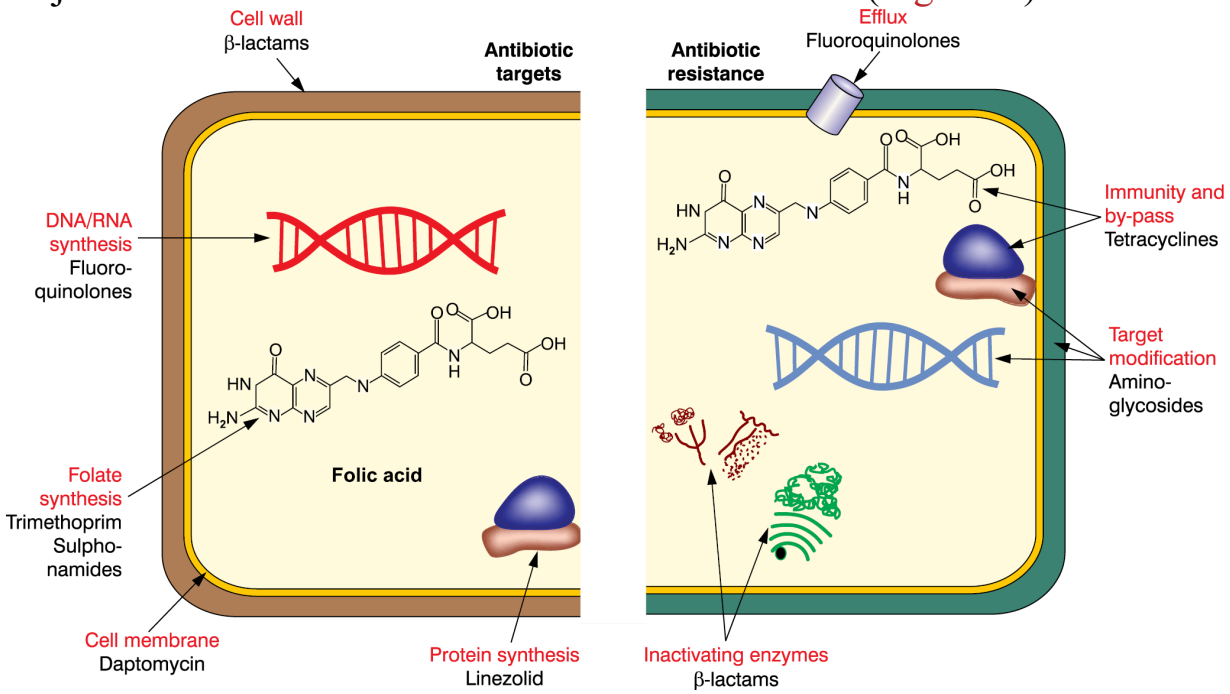


Fig. 19.1 Targets of antimicrobial action on bacterial cells and factors responsible for resistance

- Cell wall
- Cell membrane
- Nucleic acid

- Ribosome

Broad-spectrum antibiotics are active against several types of microorganisms (e.g., tetracyclines are active against gram-negative rickettsiae, mycoplasma and chlamydia) while narrow-spectrum antibiotics act against only one type of microorganism (e.g., vancomycin is active against gram-positive staphylococcus only).

Antibiotics may be **bactericidal,** that is, they kill the bacteria, or may be **bacteriostatic,** that is, they inhibit the growth of bacteria but do not kill them. In the latter type, the bacteria can grow when the drug is withdrawn. The host's immune mechanism plays a role in eliminating the bacteria.

MECHANISMS OF ACTION OF ANTIBIOTICS ON BACTERIAL CELLS

Inhibition of Cell Wall Synthesis

Penicillins and cephalosporins act by inhibiting trans-peptidase and hence inhibit cell wall synthesis. They bind to the **penicillin-binding proteins (PBP)** which are cell-surface receptors. Due to the absence of a rigid cell wall, the bacterial cell cannot withstand the osmotic pressure and hence ruptures.

Other antibiotics that act on the cell wall are:

- Carbapenems (imipenem, meropenem)
- Monobactams (aztreonam)
- Glycopeptide (vancomycin)
- Cycloserine and bacitracin

Inhibition of Protein Synthesis

Bacteria have 30S and 50S ribosomes which differ from host cell ribosomes. Protein synthesis inhibitors take advantage of this difference. They act by misreading messenger RNA and inhibiting the formation of the initiation complex.

Drugs that act on 30S ribosomes are:

- Aminoglycosides (gentamicin, amikacin)
- Tetracycline (blocks the aminoacyl transfer RNA (tRNA)

Drugs that act on 50S ribosomes are:

- Chloramphenicol, which blocks the action of peptidyl transferase, thereby preventing the formation of new peptides
- Macrolides (erythromycin, clarithromycin and azithromycin), which bind to the 50S ribosome, thereby preventing translocation
- Other antibiotics with similar action—clindamycin, streptogramin and linezolid

Inhibition of Nucleic Acid Synthesis

This may take place in three ways.

1. Inhibition of precursor synthesis:

- **Sulphonamides** These are structural analogues to *para*-amino benzoic acid (PABA) required for folic acid synthesis.
- **Trimethoprim** This inhibits dihydrofolate reductase.

2. Inhibition of DNA synthesis:

- **Fluoroquinolones** They inhibit the DNA gyrase (topoisomerase) enzyme.

3. Inhibition of mRNA synthesis:

- Rifampicin acts by blocking RNA polymerase and thereby, mRNA synthesis.

Action on Bacterial Cell Membrane

- Polymixin destroys the phospholipid component of the cell membrane and acts on *Pseudomonas* and *Acinetobacter*.

- Daptomycin disrupts the cell membrane of gram-positive cocci and acts against staphylococci and enterococci.

ANTIMICROBIAL RESISTANCE

Bacteria may be **intrinsically** resistant to one or more class of antimicrobials, e.g., *Pseudomonas* to Penicillin G.

Acquired resistance by mutation or acquisition of resistance genes from other organisms results in any of the following actions:

- Production of enzymes that destroy the antibacterial drug (e.g., beta-lactamases in penicillin and cephalosporins)
- Expression of efflux systems that prevent the drug from reaching its intracellular target [e.g., efflux pump mechanism (fluoroquinolone resistance)]
- Reduction of permeability of drug through mutation in porin proteins (e.g., aminoglycosides)
- Modification of the drug's target site (e.g., penicillin-binding protein for penicillins)
- Production of an alternative metabolic pathway that evades the action of the drug (e.g., folate metabolism)

Acquisition of new genetic material may be through:

1. **Vertical gene transfer (VGT)** Resistance genes are transferred directly to all bacterial progeny during DNA replication. A spontaneous mutation in the bacterial chromosome imparts resistance to a member of the bacterial population.
2. **Horizontal gene transfer (HGT)** Genetic material contained in small packets of DNA can be transferred between individual bacteria of the same species or even between different species by:

    - Conjugation
    - Transformation
    - Transduction

Transposons or, at times, plasmids, facilitate the incorporation of multiple

resistance genes into a host genome (see Chapter 5).

ANTIBIOTIC SENSITIVITY TESTS

Optimum therapy of bacterial infection depends on choosing the antibiotic most effective against the causative agent. Therefore, it is essential to determine the susceptibility of isolates of pathogenic bacteria to antibiotics that are likely to be used in treatment. Laboratory testing to detect susceptibility is carried out by diffusion or dilution methods (Fig. 19.2).

- Diffusion tests
  - Kirby–Bauer disc diffusion method
  - Stokes disc diffusion method
- Dilution tests
  - Broth dilution method
  - Agar dilution method

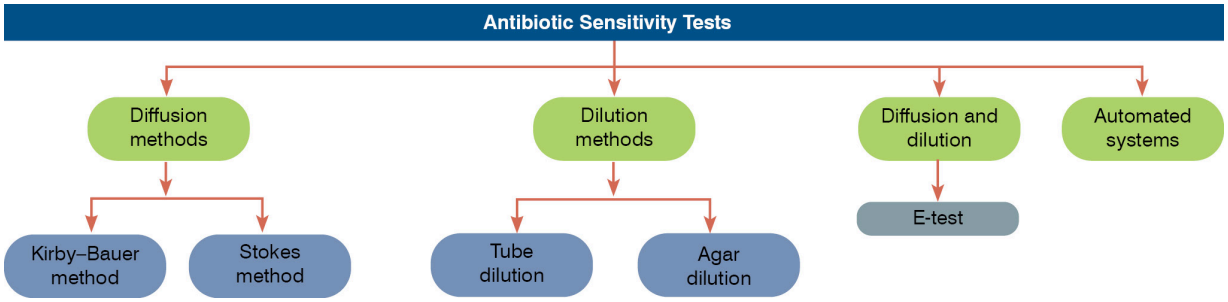


Fig. 19.2 Antibiotic sensitivity tests

Diffusion Test

Here, the drug is allowed to diffuse through a solid medium so that a gradient is established, the concentration being highest near the site of application of the drug and decreasing with distance. The test bacterium is seeded on the medium and its sensitivity to the antibiotic determined by the zone of inhibition of its growth.

The **disc diffusion** method uses filter paper discs, 6.0 mm in diameter, charged with appropriate concentrations of the antibiotic. The discs are stored in cold and dry conditions. They may be prepared in the laboratory or purchased commercially.

Kirby–Bauer method

- A suitable standard dilution of a broth culture of the test bacterium is inoculated on the surface of a solid medium [**Cation-adjusted Mueller–Hinton agar (CAMHA)**] as a lawn culture.
- After drying the plate (at 37°C for 30 minutes), antibiotic discs (4–6 per 9 cm plate) are applied with sterile forceps.
- After overnight incubation, the degree of sensitivity is determined by measuring the zones of inhibition of growth around the discs. Growth will be inhibited around the discs containing antibiotics to which the bacterium is susceptible but not around those to which it is resistant (Fig. 19.3).

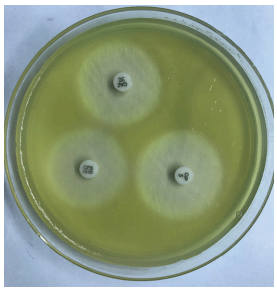


Fig. 19.3 Zone of inhibition around antibiotic discs on the lawn culture of test bacteria (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- The diameter of the zone of inhibition is influenced by a variety of factors, such as diffusibility of the drug, disc concentration, nature and composition of the medium, its thickness, presence of inhibitory or stimulatory substances, pH and time of incubation. It is also necessary to check the potency of the discs periodically using standard ATCC strains like *S. aureus* ATCC 25923, *E. coli* ATCC 25922 or *P. aeruginosa* ATCC 27853 with known zone diameters for antibiotics.
- Zones of inhibition around the discs are recorded and interpreted according to the zone diameters available in the tables of the internationally-accepted guidelines recommended by **Clinical and Laboratory Standards Institute (CLSI)**. The other

guidelines used are those of the **European Committee on Antimicrobial Susceptibility Testing (EUCAST).**

Stokes method

Comparison of the zones of inhibition between the standard and test bacteria indicates the sensitivity/resistance of the latter.

A bacterium can be:

- **Susceptible** when it is inhibited by the concentration of the drug usually achieved in the blood following administration at the recommended dosage.
- **Intermediately susceptible** when it is susceptible to the drug at higher-than-normal dosages or when the drug has clinical efficacy at the body site where the drugs are physiologically concentrated.
- **Resistant** to the drug when it is not inhibited by the drug and when specific resistance mechanisms are present in the bacterial isolate.

Choice of antibiotic disc Antibiotics for susceptibility tests should be chosen with discrimination. Only clinically relevant antibiotics should be tested, e.g., chloramphenicol need not be tested against urinary pathogens as the drug is excreted in urine mostly in the inactive form. Nitrofurantoin needs to be tested only against urinary pathogens.

E-test

A recent modification of the agar diffusion susceptibility test employing a quantitative diffusion gradient is known as the **Epsilometer** or E-test (Fig. 19.4). The **minimum inhibitory concentration (MIC)** is obtained by noting the lowest concentration of the gradient, which inhibits bacterial growth.

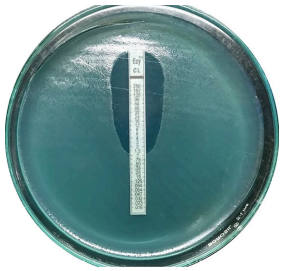


Fig. 19.4 MIC of colistin by E-test for *Pseudomonas aeruginosa*—the ellipsoidal zone of inhibition has a cut-off where the zone meets the strip incorporated with gradient concentration of the antibiotic (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Dilution Test

Here, serial dilutions of the drug are prepared and inoculated with the test bacterium. Dilution tests are generally employed when the therapeutic dose is to be regulated accurately as in the treatment of bacterial endocarditis, for slow-growing bacteria such as tubercle bacilli, and when small degrees of resistance are to be demonstrated.

Broth dilution

In this method, serial dilutions of the drug in a broth are tested against a standardised suspension of the test bacterium. After overnight incubation, the MIC is read by noting the lowest concentration of the drug that inhibits growth.

The **minimum bactericidal concentration (MBC)** is the lowest concentration of the drug that kills the bacterium, which is estimated by subculturing on solid medium.

Agar dilution

This method is more convenient when several strains are to be tested at the same time. Serial dilutions of the drug are prepared in agar and poured into plates. Many strains are inoculated on each plate containing an antibiotic dilution.

Automated Methods to Detect Antibiotic Susceptibility

Automated versions of sensitivity tests are available and are used in large laboratories for quick turnaround time.

Several automated systems have been developed to detect MICs of

antibiotics. Some of the commonly used ones are:

i) **Automated microtitre plates** These use the fluorometric plate-reading system.

ii) **VITEK system** This serves the dual purpose of identifying bacteria and determining the MIC levels of several antibiotics against the organism (Fig. 19.5).

Fig. 19.5 VITEK system for rapid identification of bacteria and determining the MIC levels of a set of antibiotics for an organism (*Source:* Centre for Virology and Molecular Biology, Pushpagiri Institute of Medical Sciences and Research Centre, Thiruvalla, Kerala)

Detection of inhibitory enzymes like the beta-lactamase produced by bacteria are used as rapid methods to detect resistance even before a sensitivity test is done. These are colourimetric tests which are rapid indicators of beta-lactamase production and its associated resistance, e.g., beta-lactamase-producing *Haemophilus*, *Moraxella* and *Neisseria* spp. and penicillinase-producing staphylococci.

Antibiotic Assays in Body Fluids

These are required to verify whether adequate drug concentrations are achieved in blood and other body fluids, and to guard against excessive blood levels of potentially toxic drugs. Some antibiotics are monitored, both to ensure adequate dosing for efficacy, and to avoid the risks associated with potentially toxic levels. Aminoglycosides and vancomycin are the most frequently used agents to require serum assays. These assays are done in the clinical biochemistry laboratory.

Antibiotics must be judiciously used to treat and prevent infectious diseases. The treatment must be based on the susceptibility pattern which is detected in vitro against the organism, the pharmacokinetics and pharmacodynamics of the antibiotic, and its safety for the patient.

ANTIBIOTIC POLICY

Antimicrobial resistance has become a matter of great concern, globally as well as in our country. Resistance has emerged even to newer, more potent antimicrobial agents like carbapenems, giving rise to **MDR (multidrug resistant)** and **pandrug-resistant isolates.** Guidelines are required for rational use of antibiotics, both at hospitals, and at the national levels. Guidelines for antibiotic use are required to ensure appropriate antimicrobial treatment and also to limit their inappropriate use in the treatment of infections. An antibiotic policy guides the treating physician on the most appropriate antibiotic to be used empirically (before the culture and sensitivity report) in a particular infection. This is based on the data of susceptibility patterns of common pathogens causing infection in the hospital over a period of time. An antibiotic policy helps in antibiotic selection and deciding on dosing, route, duration, emergence of resistance and cost. The policy also must mention the need to switch to specific antibiotics following culture and sensitivity and the dose and duration of such treatment.

Antibiotic policy is periodically reviewed and updated to keep up with emerging resistance patterns in the hospital. A **National Guideline (Treatment Guidelines for Antimicrobial use for Common Syndromes-2017)** has been formulated by the Indian Council of Medical Research, Department of Health (Ministry of Health and Family Welfare, Government of India) based on the national data.

Antibiotic Stewardship

- To control antibiotic resistance, hospitals have introduced antibiotic stewardship programmes. Methicillin-resistant *Staphylococcus aureus* (MRSA), beta-lactamase-producing gram-negative bacteria, ESKAPE organisms (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) are responsible for the majority of health care-associated infections. Antibiotic stewardship programmes in hospitals aim to reduce inappropriate treatment by educating

prescribers to practice evidence-based prescribing, which will result in reducing the inappropriate use of antibiotics.
- Targeted treatment using narrow-spectrum antibiotics (thereby preserving high-end broad-spectrum antibiotics).
- Limiting the adverse effects of antibiotics.

Some of the core elements of the antibiotic stewardship programme are:

- Requirement by the management to implement the programme by allotting money, personnel and other facilities
- A physician as the leader of the team to effectively monitor appropriate therapy
- A pharmacist in the team to improve antibiotic dispensation and use and to monitor the quantity of antibiotics used per patient
- Evaluating the need for treatment after a set period of initial treatment, called the 'antibiotic time-out' after 48 hours
- Tracking—monitoring antibiotic prescription and resistance patterns
- Reporting—regular reporting of information on antibiotic use and resistance to doctors, nurses and relevant staff
- Education—educating clinicians about resistance and optimal prescribing

Implementing an antibiotic stewardship programme helps patient safety by improving antibiotic use and controlling resistance, thus resulting in best practice.

Guidelines for restricting emergence of antibiotic resistance

- Avoiding unnecessary use (viral upper respiratory infections, pharyngitis, viral gastroenteritis, etc.)
- Rational use in hospitals and health care settings
- Prevention of over-the-counter sales in pharmacies without prescription
- Restricting the use of antibiotics in animal feeds and industrial sectors

The general principles of an antibiotic policy of a hospital are:

- Monitoring antibiotic use in hospitals
- Restricting use of third- and fourth-generation antibiotics
- Rational empiric therapy based on susceptibility data from microbiology department
- Implementing antibiotic stewardship in critical care units

Key points

- There are four major sites on the bacterial cell that antibiotics act on: (i) cell wall, (ii) cell membrane, (iii) nucleic acid and (iv) ribosome.
- Antibiotics may be bactericidal, that is, capable of killing bacteria, or bacteriostatic, wherein they inhibit their growth but do not kill them.
- Antibiotics act by:
    - Inhibiting cell wall synthesis either by inactivating the antibiotics by enzymes, e.g., beta-lactamases, or by altering the protein on the site of binding of the antibiotic, e.g., penicillin-binding protein (PBP)
    - Inhibiting protein synthesis by binding to the 30S and 50S ribosomes
    - Inhibiting nucleic acid synthesis by inhibiting the gyrase enzyme or folic acid synthesis
    - Acting on the cell membrane to destroy the phospholipid component
- Bacteria may be intrinsically resistant to one or more classes of antimicrobials, e.g., *Pseudomonas* to penicillin G.
- They may acquire resistance by mutation or acquisition of resistance genes from other organisms.
- Bacteria may acquire resistance to certain antibiotics, following which, they may:
    - Produce enzymes that destroy the antibacterial drug (e.g., carbapenemase enzyme by *Pseudomonas* inactivates imipenem)
    - Express efflux systems that prevent the drug from reaching its intracellular target (e.g., ciprofloxacin resistance in *Salmonella*)
    - Modify the drug's target site (e.g., altered penicillin-binding protein (PBP) site in pneumococci, causing resistance to penicillin)
    - Produce an alternative metabolic pathway that evades the action of the drug (e.g., trimethoprim resistance in bacteria by the alteration of the folate pathway)
- Genetic transfer of resistance mechanisms may be by:
    - Vertical gene transfer (VGT) Resistance genes are transferred directly to all bacterial progeny during DNA replication.
    - Horizontal gene transfer (HGT) Genetic material contained in small packets of DNA can be transferred between individual bacteria of the same species or even between different species by:

      – Conjugation

      – Transformation

      – Transduction
- There are two types of antibiotic susceptibility tests:
    - **Diffusion tests**

      a) Kirby Bauer disc diffusion

      b) Stokes' method

c) E-test by gradient diffusion

- **Dilution tests**

  a) Broth dilution tests to determine the minimum inhibitory concentration and minimum bactericidal concentrations

  b) Agar dilution methods

- **Automated methods** to test antibioteic suscettibility include VITEK, Phoenix and MicroScan WalkAway which directly provide the minimal inhibitory concentrations.
- Antibiotics for susceptibility tests should be clinically relevant. Susceptibility tests should be done only with known or presumed pathogens.
- A recent modification of the agar diffusion susceptibility test is the Epsilometer test.
- The MIC of the drug against the bacterium is determined by noting the lowest concentration at which growth is inhibited.
- The agar dilution method is more convenient for simultaneously testing several bacterial strains on agar plates containing different concentrations of the drug.
- Antibiotic assays in body fluids are required to verify whether adequate drug concentrations are achieved in blood and other body fluids.
- Antibiotic policy and national guidelines are required for rational antibiotic use to restrict emergence of MDR and pandrug-resistant isolates.

Questions

Short Answers

1. Mechanisms of antibiotic action on bacteria
2. Mechanisms of antibiotic resistance
3. Rational antibiotic use
4. Antibiotic policy
5. Antibody stewardship

Part III: Bacteriology

Chapter 20

Staphylococci

Chapter navigation

- *S. aureus*
- Coagulase-negative staphylococci
- Micrococci

INTRODUCTION

Staphylococci are gram-positive cocci that occur in clusters. *Staphylococcus aureus* is the most important human pathogen, commonly causing localised suppurative lesions in humans (*Clinical case* 1) as well as disseminated infections. Its ability to develop resistance to penicillin and other antibiotics makes infections caused by *S. aureus* difficult to treat. Other clinically important species, collectively known as **coagulase-negative staphylococci (CoNS)** include *S. epidermidis, S. lugdunensis, S. haemolyticus* and *S. hominis*. They are found as commensals on the skin but they can cause health care-associated infections (HAI) in a compromised host.

S. saprophyticus causes urinary tract infections in young women. The other human pathogen, *S. saprophyticus,* is similar to *S. epidermidis*, except that the former is resistant to novobiocin, whereas the latter is not.

Clinical Case 1

A 56-year-old man sustained an abrasion on his hand while working in the garden. Three days later, he developed a painful localised swelling with purulent discharge from the lesion. A Gram stain of the discharge showed gram-positive cocci in clusters. Culture of the pus showed golden-yellow hemolytic colonies on blood agar, which were identified as S. aureus. Antimicrobial susceptibility showed the organism to be sensitive to penicillin, cefazolin, clindamycin and oxacillin. The wound healed after the pus was drained. The patient did not require any antibiotics.

STAPHYLOCOCCUS AUREUS

Staphylococci are spherical cocci, approximately 1 ∝m in diameter, arranged characteristically in grape-like clusters (Fig. 20.1). Cluster formation is due to cell division occurring in three planes, with daughter cells tending to remain in close proximity.

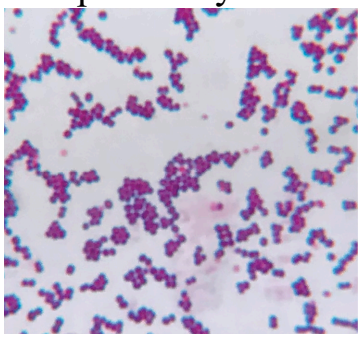


Fig. 20.1 Gram-positive cocci in clusters: *Staphylococcus aureus* (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Staphylococci produce two types of diseases:

- **Localised or disseminated infections** *S. aureus* gains access to damaged skin, mucosal or tissue sites, colonises by adhering to cells or extracellular matrices, evades host defence mechanisms, multiplies and causes tissue damage.
- **Toxin-mediated infections** Some cases of food poisoning and antibiotic-associated diarrhea are caused by enterotoxin-forming staphylococci. The toxin also exhibits pyrogenic, mitogenic, hypotensive, thrombocytopenic and cytotoxic effects (details discussed subsequently).

Virulence Factors

The four major virulence factors of staphylococci include cell-associated and cell-surface proteins, extracellular enzymes and toxins (Table 20.1).

Table 20.1 Staphylococcal virulence factors and their constituents

Virulence factor	Constituents
Cell-associated factors	Polysaccharide peptidoglycan
	Teichoic acid
Cell-surface proteins	Protein A
	Clumping factor

Extracellular enzymes	Coagulase
	Lipid hydrolases
	Hyaluronidase
	Nuclease
Toxins	Cytotoxin
	Alpha-hemolysin
	Beta-hemolysin
	Gamma-hemolysin
	Delta-hemolysin
	Panton–Valentine leucocidin (PVL)

Regulation of virulence factors

A number of staphylococcal factors, both cell-associated and extracellular, which may influence virulence have been identified. The production of virulence factors is regulated by complex regulatory mechanisms through a set of genes which express by binding of ligands to host cell receptors.

I. Cell-associated factors

a. The cell wall **polysaccharide peptidoglycan** confers rigidity and structural integrity to the bacterial cell. It activates the complement and induces the release of inflammatory cytokines.
b. **Teichoic acid**, an antigenic component of the cell wall, facilitates adhesion of the cocci to the host cell surface and protects them from complement-mediated opsonisation.
c. **Capsular polysaccharide** surrounding the cell wall inhibits opsonisation.

II. Cell-surface proteins

Protein A, present in most *S. aureus* strains, has many biological properties, including chemotactic, anti-phagocytic and anti-complementary effects. It also induces platelet damage and hypersensitivity. Protein A binds to the *Fc* terminal of IgG molecules (except IgG3), leaving the *Fab* region free to combine with its specific antigen; it is often used in **staphylococcal co-agglutination** tests. Protein A is a B cell mitogen. It has also been used as a ligand for the isolation of IgG.

Clumping factor, another surface protein known as **bound coagulase**, is

responsible for the slide coagulase test.

Staphylococci possess **protein receptors** for many mammalian proteins such as fibronectin, fibrinogen, IgG and C1q. These facilitate staphylococcal adhesion to host cells and tissues.

III. Extracellular enzymes

a. **Coagulase** This is an enzyme which acts with a coagulase-reacting factor (CRF) present in plasma, to bind to prothrombin and convert fibrinogen to fibrin (basis for tube coagulase test).

b. **Lipid hydrolases** Staphylococci produce a number of lipases, which help them infect the skin and subcutaneous tissues.

c. **Hyaluronidase** It breaks down the connective tissue. Staphylokinase (fibrinolysin) and proteases help in the spread of infection.

d. **Nuclease** This heat-stable DNase is a component of *S. aureus*, which helps in the identification of the organism.

IV. Toxins

Cytolytic toxins are membrane-active substances, consisting of four hemolysins and a leucocidin.

a. **Alpha-hemolysin** (alpha toxin, lysin) is the most important among the cytolytic toxins. It is a protein inactivated at 70°C but reactivated paradoxically at 100°C. This is because at 60–70°C, the toxin combines with a heat-labile inhibitor which is denatured at 100°C, leaving the toxin free. Alpha toxin lyses rabbit erythrocytes. It is also **leucocidal, cytotoxic** and **dermonecrotic** (on intradermal inoculation in rabbits). It is toxic to macrophages, lysosomes, muscle tissues, the renal cortex and the circulatory system.

b. **Beta-hemolysin** is a sphingomyelinase, which is hemolytic for sheep cells but not for human or rabbit cells. It exhibits a **hot–cold phenomenon,** the hemolysis being initiated at 37°C but becoming evident only after chilling.

c. **Gamma-hemolysin** is composed of two separate proteins, both of which are necessary for hemolytic activity.

d. **Delta-hemolysin** has a detergent-like effect on the cell membranes of erythrocytes, leucocytes, macrophages and platelets.

e. **Panton–Valentine leucocidin** (called **PVL**) is also a two-component toxin, like gamma-lysin, composed of the S and F components. Such bi-component, membrane-active toxins have been grouped as **synergohymenotropic toxins**. The interest in PVL has been renewed because of its increased association with community-acquired MRSA (methicillin-resistant *S. aureus*).

Clinical Case 2

A 15-year-old boy began vomiting 6–7 hours after consuming a pineapple pastry at a birthday party. He passed loose stools but there was no fever. Six other children who had attended the party reported a similar illness. On further questioning, it was found that the common dish that all the affected children had consumed was the pastry. The cream from the pastry tested positive for S. aureus*. Further investigations found that a specimen obtained from the nose of the cook was positive for* S. aureus*. Pulse field gel electrophoresis ascertained both to be of the same type, which indicated a common source. The cook was treated for the carrier state with local antibiotic application. The children recovered within a day or two and did not require any antibiotics.*

Enterotoxin is a potent staphylococcal exotoxin which causes food poisoning resulting in nausea, vomiting and diarrhea 2–6 hours after consuming food contaminated by the preformed toxin. The toxin is relatively heat-stable, resisting 100°C for 10 to 40 minutes. Meat and fish or milk and milk products cooked and left at room temperature after contamination with staphylococci are the common food items responsible for the infection. **The source of infection is usually a food handler who is a carrier**. The illness is often self-limiting, with recovery in a day or two (*Clinical case 2*)**.** Eight antigenic types of enterotoxin are currently known —**enterotoxins *A, B,* C_{1-3}*, D, E* and *H*.** The toxin is believed to act directly on the autonomic nervous system to cause the illness, rather than on the gastrointestinal mucosa. **The toxin is antigenic and is neutralised by the specific antitoxin.** Type A toxin is responsible for most cases. Sensitive serological tests such as latex agglutination and ELISA are available for the detection of the toxin.

Pathogenesis

Localised infections

Staphylococcal infections are among the most common bacterial infections and range from the mild to the fatal (Table 20.2). They are characteristically

localised pyogenic lesions (Fig. 20.2), in contrast to the spreading nature of streptococcal infections. *S. aureus* gain access to damaged skin, mucosal or tissue sites, colonise by adhering to cells or extracellular matrix, evade host defence mechanisms, multiply and cause tissue damage. The prototype of localised infection is an abscess or a furuncle. They may cause pyoderma or impetigo. Dermonecrotic factor released by the organism causes necrosis of the tissue in a hair follicle. There is production of coagulase, which coagulates the fibrin and localises the lesion, attracting inflammatory cells. Later, the necrotic tissue liquefies. The abscess points to the surface. If it is not drained, fibrosis occurs with granulation tissue filling the cavity with eventual healing.

Infection might disseminate from a focus and spread via the lymphatics and bloodstream to distant organs, causing osteomyelitis, empyema, pneumonia, meningitis, endocarditis or sepsis.

Fig. 20.2 Erythematous lesions with superficial ulceration due to *Staphylococcus aureus* (*Source:* Public Health Image Library, ID 7826 / Bruno Coignard, M.D.; Jeff Hageman, M.H.S. / CDC)

Table 20.2 Staphylococcal diseases

Skin and soft tissue	Folliculitis, furuncle (boil), abscess (particularly breast abscess), wound infection, carbuncle, impetigo, paronychia and less often, cellulitis
Musculoskeletal system	Osteomyelitis, arthritis, bursitis and pyomyositis
Respiratory system	Tonsillitis, pharyngitis, sinusitis, otitis, bronchopneumonia, lung abscess, empyema and rarely, pneumonia
Central nervous system	Abscess, meningitis and intracranial thrombophlebitis
Endovascular	Bacteremia, septicemia, pyemia and endocarditis

Urinary	Infection in association with local instrumentation, implants or diabetes
Common toxin-mediated staphylococcal diseases	Food poisoning Toxic shock syndrome Scalded skin syndrome

Toxin-mediated infections

As mentioned earlier, a certain group of infections caused by *S. aureus* are toxin-mediated. Organisms may or may not be able to be isolated from the sites of toxin action.

Some cases of post-antibiotic diarrhea are caused by enterotoxin-forming staphylococci. The toxin also exhibits pyrogenic, mitogenic, hypotensive, thrombocytopenic and cytotoxic effects.

Toxic shock syndrome toxin (TSST) (formerly, enterotoxin F) causes a potentially fatal multisystem disease called **toxic shock syndrome (TSS)** presenting with fever, hypotension, myalgia, vomiting, diarrhea, mucosal hyperemia and an erythematous rash which desquamates subsequently. This is caused by TSST-producing *S. aureus* strains usually belonging to bacteriophage group I.

Though initially related to tampon use TSS is now rare in tampon users. The syndrome occurs in other infections of the skin, mucosa and also in some surgical wounds.

> Staphylococcal enterotoxins and TSST-1 are **superantigens** which are potent activators of T lymphocytes. Being Vb-restricted T cell mitogens, they stimulate large numbers of T cells. This leads to an excessive and dysregulated immune response, with release of interleukins 1 and 2, tumour necrosis factor and interferon gamma.

An unrelated disease of unknown etiology, Kawasaki disease, clinically resembles TSS and is caused by the superantigen of *S. aureus*.

Exfoliative (epidermolytic) toxin, also known as ET or **exfoliatin**, is responsible for the **staphylococcal scalded skin syndrome (SSSS)**, an exfoliative skin disease in which the outer layer of the epidermis gets separated from the underlying tissues. The severe form of SSSS is known as **Ritter's disease** in the newborn and **toxic epidermal necrolysis** in older patients. Milder forms are **pemphigus neonatorum** and **bullous impetigo.**

Epidemiology

Sites Staphylococci are primary pathogens of human beings and animals, colonising the skin, apocrine glands and mucous membranes.

Source The most common sources of infection are human patients and carriers; animals and inanimate objects are less important. Patients with superficial infections and respiratory infections disseminate large numbers of staphylococci into the environment. About 10–30 per cent of healthy persons carry staphylococci in their nose and about 10 per cent in the perineum and also on their hair. Vaginal carriage is about 5–10 per cent, which rises greatly during menses, a factor relevant in the pathogenesis of TSS related to menstruation.

Staphylococcal carriage starts early in life, colonisation of the umbilical stump being very common in babies born in hospitals.

Shedders
Some carriers, called 'shedders', disseminate very large numbers of cocci for prolonged periods. The cocci shed by patients and carriers contaminate fomites such as handkerchiefs, bed linen and blankets and may persist on them for days or weeks.

Staphylococci may also come from infected domestic animals such as cows.

Antibiotic Resistance

Staphylococci are among the more resistant bacteria.

Types of resistance

Beta-lactamase-mediated

This is mediated through the production of β-lactamase or **penicillinase**, which inactivates penicillin by splitting the beta-lactam ring. These are inducible enzymes that are plasmid-mediated. They can be transmitted by transduction or conjugation. **Penicillinase-producing strains remain sensitive to penicillinase-resistant penicillins such as methicillin and cloxacillin while the β-lactamase-producing strains are sensitive to beta-lactamase inhibitor combinations like amoxicillin–clavulanic acid**.

Methicillin resistance (MRSA)

Alteration at the target penicillin-binding site PBP2a causes changes in bacterial surface receptors, thereby reducing the binding affinity of beta-

lactam antibiotics to cells. This mechanism imparts resistance to all beta-lactam antibiotics. This is regulated by a set of chromosomal genes called **staphylococcal cassette chromosomal *mec* genes (SCC*mec*)**, especially the *mecA* gene. Development of resistance also occurs through several steps of structural diversification of the non-binding domain of native PBPs, changes in the promoters of ***mecA*** homologues and acquisition of SCC*mec* gene. Based on the type of these genes, the MRSA strains are classified as:

a) ***Hospital-acquired MRSA (HA-MRSA)*** Here, the SCC*mec* is predominantly of the types I, II and III; these are multidrug-resistant.

b) ***Community-acquired MRSA (CA-MRSA)*** SCC*mec* is predominantly type IV. These strains are less resistant, more likely to produce PVL toxin and are more transmissible.

A majority of these strains also show resistance to other antibiotics like erythromycins, tetracyclines and aminoglycosides and cause outbreaks of hospital infection. **HA-MRSA** and **CA-MRSA** are a public health concern now as the distinction between the two is getting blurred and both the types are circulating in the community as well as hospitals.

Vancomycin resistance (VRSA)

Strains showing resistance to vancomycin have started to emerge in some parts of the world. These strains have been shown to be harbouring both *vanA* and *mecA* genes. This is the cause of major therapeutic challenges worldwide as these organisms remain susceptible only to linezolid as of now.

Vancomycin intermediate resistance (VISA)

These have decreased susceptibility to vancomycin and do not carry any resistance genes. Instead, they have a thickened cell wall. These organisms have been isolated from patients who are on prolonged vancomycin treatment but show clinical failure to treatment with vancomycin. Terms like **hVISA** have been used for the heterogeneously resistant population of *S. aureus,* which is a likely step towards full VISA development. **Health care-associated (HAI) infections** by staphylococci are caused by strains resistant to various antibiotics.

Epidemic methicillin-resistant *S. aureus* strains, also called **EMRSA**, get easily disseminated in a hospital environment and are responsible for postoperative wound infections and other cross-infections in the hospital.

Outbreaks of MRSA are seen in hospitals. Such events need to be identified early in order to undertake interventions for early control of spread (*see* Chapter 60).

Detection of MRSA This is done by **cefoxitin disc diffusion agar** as per the present international guidelines. Cefoxitin resistance correlates with the presence of *mecA* gene.

Oxacillin screen agar Some strains called **borderline resistant *S. aureus* (BORSA)** have been found, which are missed by cefoxitin susceptibility test and may need to be tested under special incubation conditions using **oxacillin screen agar**. Oxacillin powder is incorporated into Mueller Hinton Agar (MHA). Suspected colonies are spot-inoculated along with standard positive ATCC 38591 MRSA and an MSSA—ATCC 29213. The plates are read after incubating them at 35–37°C for 18–24 hours. These strains that grow are resistant to penicillins such as methicillin, oxacillin and nafcillin.

Treatment

For nasal carriers, treatment with local application of **mupirocin** is required. **Chlorhexidine** may be needed in some carriers or colonisers as a small number of strains have shown resistance to mupirocin. In resistant cases posing major problems, rifampicin along with another oral antibiotic may be effective in the long-term suppression or elimination of the carrier state.

Typing of Staphylococci

Typing may be done if information is desired for epidemiological purposes. The two major techniques are:

- **Phenotypic**—based on phenotypic characters like antibiograms, phage types, biotypes, etc.
- **Genotypic**—based on the genetic composition like rRNA or pulse field gel electrophoresis (PFGE) or by random amplification of polymorphic DNA (RAPD) method

Phenotypic–bacteriophage typing

Staphylococci may be typed based on their susceptibility to bacteriophages (Fig. 20.3). In India, the phage typing centre at Maulana Azad Medical College, New Delhi, found that phage group III was predominant among MRSA strains, whereas MSSA from the community belonged to phage group NA (phage type 81).

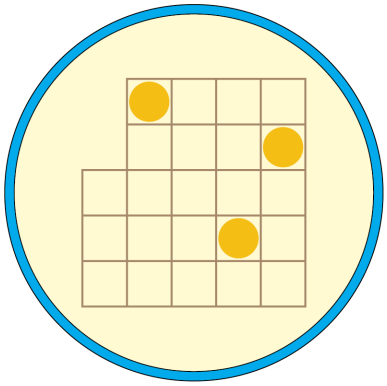


Fig. 20.3 Bacteriophage typing of staphylococci

Molecular typing

DNA fingerprinting using **RFLP**, random amplification of polymorphic DNA (RAPD) and **ribotyping** are **PCR-based** methods that are used to type staphylococci. **PFGE** and sequence-based typing methods **(MLST)** are now used in outbreak investigations as they are more discriminatory. These methods are described in Chapter 6.

Laboratory Diagnosis of Staphylococcal Infections

1. **Specimen** The specimens to be collected depend on the type of lesion. For the detection of carriers, nasal swab, axillary and perineal swabs are collected.

2. **Microscopy** Gram-stained smears are useful in the case of pus, where cocci in clusters may be seen.

3. **Culture** The specimens are plated on **blood agar** on selective media like **Ludlam's** or **salt-milk agar** or **Robertson's cooked meat medium** containing 10% sodium chloride. Smears from the cultures are examined and the coagulase test carried out when staphylococci are isolated.

Cultural characteristics Staphylococci grow readily on ordinary media within a temperature range of 10°C to 42°C, the optimum being 37°C, and a pH of 7.4–7.6. They are aerobes and facultative anaerobes.

- **On blood agar** Most *S. aureus* strains are hemolytic, especially when incubated under 20–25% carbon dioxide. **Hemolysis** is marked on rabbit or sheep blood and weak on horse blood agar. For primary isolation, sheep blood agar is recommended. Human blood should not be used as it may contain antibodies or other inhibitors (Fig. 20.4).

Fig. 20.4 Growth of *Staphylococcus aureus* on blood agar showing golden-yellow hemolytic colonies (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- **On nutrient agar** Pigment production occurs optimally at 22°C and only in aerobic cultures. Pigment production is enhanced when l% glycerol monoacetate or milk is incorporated in the medium.
- **On selective media** These may be needed for isolating *S. aureus* from specimens such as feces containing other bacteria. These include media containing 8–10% NaCl (**salt-milk agar**, **salt broth**), lithium chloride and tellurite (**Ludlam's medium**) and polymyxin.

4. Identification It is based on coagulase test.

Coagulase test The coagulase test can be done using two methods: tube and slide (Table 20.3 and Fig. 20.5a, b).

Table 20.3 Difference between detection of free coagulase (tube coagulase) and clumping factor (slide coagulase) tests

Tube method to detect free coagulase	Slide method to detect bound coagulase (clumping factor)
There is activation of plasma coagulase-reacting factor (CRF) found in rabbit plasma to form a coagulase–CRP complex.	This is bound to the bacterial cell wall and reacts directly with fibrinogen of human or rabbit plasma, resulting in the alteration of

This complex, in turn, reacts with fibrinogen to produce the fibrin clot.	fibrinogen which precipitates on the staphylococcal cell, causing the cells to clump when a bacterial suspension is mixed with plasma. This does not require coagulase-reacting factor.
Procedure 0.1 mL of a young broth culture or agar culture suspension of the isolate is added to about 0.5 mL of heparinised or oxalated human or rabbit plasma. The tubes are incubated in a water bath at 37°C for 3–6 hours to demonstrate clotting.	**Procedure** A drop of human or rabbit plasma is added to an emulsion of the isolate in a drop of saline and mixed.
Result The clotting of plasma is a positive test.	**Result** Prompt clumping of the cocci indicates a positive test.

The **tube coagulase test** detects the free coagulase (an extracellular product) of *S. aureus* (Fig. 20.5a).

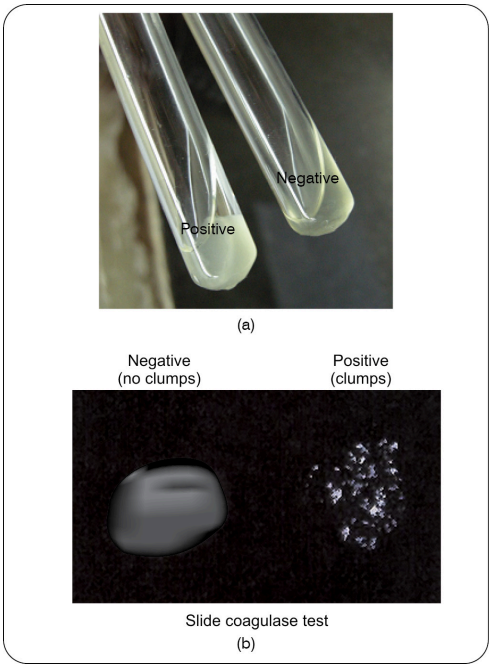


Fig. 20.5 (a) Free coagulase (tube coagulase) test: positive and negative and (b) clumping factor (slide coagulase) test: negative and positive

In the clinical laboratory, it is often necessary to differentiate *S. aureus* from *S. epidermidis* as the former is pathogenic, whereas the latter is a commensal which may contaminate the specimen. Some of the salient differences are shown in Table 20.4.

Table 20.4 Differences between *S. aureus* and *S. epidermidis*

Test	*S. aureus*	*S. epidermidis*
Coagulase	+	–
Mannitol fermentation	+	–
Heat-stable nuclease	+	–
Phenolphthalein phosphatase	+	–
Beta-hemolysis on blood agar	+	–
Golden yellow pigment	+	–
Sensitivity to lysostaphin	+	–

5. **Antimicrobial susceptibility tests** This is important as staphylococci readily develop resistance to antibiotics.

- ***D-test*** This is used to detect inducible clindamycin resistance. In this test, a clindamycin disc is placed near an erythromycin disc. If the zone of inhibition shows flattening towards the erythromycin disc, the test is positive. In such situations, clindamycin should be reported as resistant, as clinical resistance is likely to be induced while the patient is on treatment.
- ***Vancomycin susceptibility*** The current recommendation is to test using only the MIC for determining the susceptibility of *S. aureus.*

The guidelines on antimicrobial susceptibility tests are regularly revised and need to be consulted regularly. The interpretations need to be revised accordingly.

6. **Molecular diagnosis** These tests are described under typing methods and are used to detect the spread of a strain during suspected outbreaks.

Treatment

As drug resistance is very common among staphylococci, the appropriate antibiotic should be chosen based on antibiotic sensitivity tests.

- ***Benzyl penicillin*** This is the most effective antibiotic if the strain is non-penicillinase-producing. Penicillin is the drug of choice for community-acquired methicillin-sensitive disseminated staphylococcal infection. Caution must be exercised when treating patients prone to hypersensitivity reaction to penicillin. Mild infections caused by MSSA can be treated with first- or second-generation cephalosporins (cefazolin, cephalexin

or cefuroxime). However, most staphylococcal isolates from clinical material have acquired resistance to penicillin.

- ***Cloxacillin*** Methicillin was the first compound developed to combat resistance due to penicillinase (beta-lactamase) production by staphylococci. Due to limitations in the clinical use of methicillin, cloxacillins are used instead against penicillinase-producing strains to treat bone and skin infections.
- ***Vancomycin or teicoplanin*** Wherever MRSA strains are detected, vancomycin is the drug of choice. It is recommended in bloodstream infections, osteomyelitis and other deep-seated or life-threatening infections in critically ill patients. Strains resistant to vancomycin and teicoplanin are still not common and not yet reported from India.
- ***Linezolid*** This is the treatment of choice for patients infected with VRSA. This has good tissue penetration, hence can be used in skin and soft tissue infection caused by MRSA.
- For mild superficial lesions, systemic antibiotics may not be necessary. Topical application of drugs not used systemically, such as mupirocin, fusidic acid, bacitracin or chlorhexidine, may be sufficient.

OTHER COAGULASE-POSITIVE STAPHYLOCOCCI (COPS)

Besides *S. aureus*, there are other coagulase-positive staphylococci, for example, *S. intermedius* and *S. hyicus*. These are animal parasites. They rarely infect humans.

COAGULASE-NEGATIVE STAPHYLOCOCCI (CONS)

Coagulase-negative staphylococci constitute a major component of the normal flora of the human body. Some species can produce human infections—*S. epidermidis, S. haemolyticus* and *S. lugdunensis,* especially in device-associated and health care-associated infections.

S. epidermidis is the most common cause of CoNS infections in humans. It is invariably present on normal human skin. It can cause disease when the

host defences are breached. It is a common cause of **stitch abscess**. It has a predilection for growth on **implanted foreign bodies such as artificial heart valves, shunts, intravascular catheters and prosthetic appliances, leading to bacteremia**. Hospital strains of *S. epidermidis* are **usually multidrug-resistant**. It can cause cystitis and **central-line-associated bloodstream infection (BSI)**. Endocarditis may be caused, particularly in drug addicts.

Biofilm formation is an important factor in the pathogenesis of infections by *S. epidermidis*. It is an extracellular polysaccharide matrix which protects bacteria from antibacterial agents and helps in colonisation and resistance of infections.

S. saprophyticus may be present on normal human skin and the periurethral area and can cause urinary tract infection, particularly in sexually active young women. The infecting strains are usually sensitive to most common antibiotics, except nalidixic acid. *S. saprophyticus* is identified by its novobiocin resistance.

MICROCOCCI

These are gram-positive cocci which can be mistaken for *S. aureus* in culture but are larger and mostly arranged in pairs, tetrads or irregular clusters. In cultures, they form smaller colonies. They are catalase- and modified oxidase-positive and oxidative in Hugh and Leifson's oxidation–fermentation test. They are ordinarily non-pathogenic but in rare cases, cause infections in an immunocompromised host.

Key points

- *Staphylococci* are gram-positive cocci, non-motile, facultative anaerobes, and catalase-positive.
- The coagulase test is used to broadly differentiate staphylococci into coagulase-positive (*Staphylococcus aureus*) and coagulase-negative (*Staphylococcus epidermidis, Staphylococcus saprophyticus*, among others) strains.
- *Staphylococcus aureus* is a major pathogen responsible for causing suppurative infections.
- *Staphylococci* possess many virulence factors—cellular and extracellular—enzymes and toxins that cause various lesions based on their ability to invade breaks in the body's defences.

- *Staphylococcus epidermidis* forms biofilms on medical devices and causes infections associated with implants such as central venous catheters, cerebrospinal fluid shunts and intraocular lenses.
- Staphylococci are usually susceptible to penicillinase-resistant penicillins, such as methicillin and cloxacillin, and to aminoglycosides and macrolides.
- Methicillin-resistant HA-MRSA and CA-MRSA, VRSA and VISA are posing major therapeutic challenges in the treatment of these infections.

Questions

Essays

1. Describe the virulence factors of *Staphylococcus aureus* and their mechanism of action.
2. What is toxic shock syndrome? Describe its etiology and pathogenesis.

Short Answers

1. Co-agglutination (definition)
2. Differences between Staphylococcus aureus and Staphylococcus epidermidis
3. Principle and interpretation of the coagulase test
4. MRSA
5. Staphylococcal food poisoning

CHAPTER 21

Streptococci, Enterococci and Pneumococci

Chapter navigation

- Streptococci
- Enterococci
- Pneumococci

STREPTOCOCCI

Streptococci are gram-positive cocci arranged in chains or pairs (Fig. 21.1). They are part of the normal flora of humans and animals. Some of them are human pathogens. **The most important streptococcus is *Streptococcus pyogenes,* which causes pyogenic infections, with a characteristic tendency to spread, as opposed to staphylococcal lesions, which are typically localised.** Streptococci are also responsible for non-suppurative lesions, acute rheumatic fever and glomerulonephritis, which occurs as sequelae to infection.

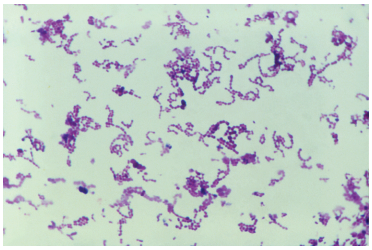


Fig. 21.1 Streptococci (gram-positive cocci in chains) (*Source:* Public Health Image Library, ID 4347 / Dr. Gilda Jones / CDC)

CLASSIFICATION

Several systems of classification have been used in medical bacteriology (Fig. 21.2).

1. **Based on oxygen requirement**, they are classified as:

- Facultative anaerobes
- Obligate anaerobes

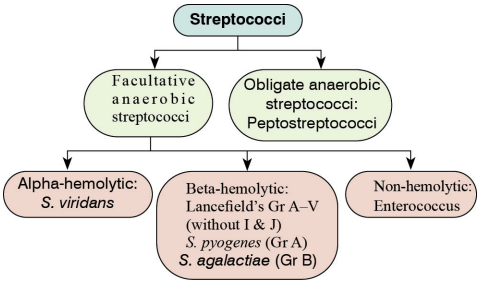

Streptococci
Facultative anaerobic streptococci
Obligate anaerobic streptococci: Peptostreptococci
Alpha-hemolytic: S. viridans
Beta-hemolytic: Lancefield's Gr A–V (without I & J) S. pyogenes (Gr A) S. agalactiae (Gr B)
Non-hemolytic: Enterococcus

Fig. 21.2 Classification of streptococci

2. **Based on hemolysis on blood agar**, that is, based on the type of hemolysis produced on sheep or horse blood agar, streptococci have been classified as α- and β-hemolytic and non-hemolytic (Fig. 21.3).

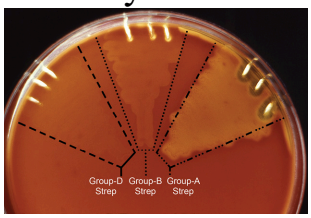


Fig. 21.3 Hemolysis on blood agar (*Source:* Public Health Image Library, ID 10861 / Dr. Richard Facklam / CDC)

- **Alpha (α)-hemolytic streptococci** These produce a greenish discolouration with partial hemolysis around the colonies. The zone of lysis is small (1 or 2 mm wide). Most alpha-hemolytic streptococci are normal commensals in the throat, and may cause opportunistic infections rarely. These include the viridans group of streptococci and pneumococcus.
- **Beta (β)-hemolytic streptococci** These produce a sharply defined, clear, colourless zone of complete hemolysis, 2–4 mm wide. The term 'hemolytic streptococci' applies strictly only to beta-hemolytic strains. Most pathogenic streptococci belong to this group.
- **Non-hemolytic streptococci** These do not produce hemolysis in the medium and include mostly the fecal streptococci, which

are currently classified as a separate species, the ***Enterococcus***.

3. **Based on carbohydrate antigen (C) on the cell wall**, Rebecca Lancefield (1933) serologically grouped hemolytic streptococci into what are known as Lancefield's groups, twenty of which have been identified so far and named A–H and K–V (without I and J) (Table 21.1).

Table 21.1 Medically important streptococci and their characteristics

Species or common name	Lancefield group	Hemolysis	Habitat in human hosts	Laboratory tests	Common diseases caused
S. pyogenes	A Griffith's M-types (80) Nephritogenic pyoderma: M types 2, 49, 57, 59, 60, and 61	Beta	Throat, skin	Bacitracin-sensitive; PYR test-positive; Ribose not fermented	Upper respiratory tract infections, impetigo, pyoderma, Neonatal meningitis, septicemia Sequelae: Rheumatic fever glomerulonephritis
S. agalactiae	B	Beta	Female genital tract, rectum	cAMP test, hippurate hydrolysis	Neonatal infections: meningitis or septicemia Adults: Sepsis, wound infections
S. equisimilis	C	Beta	Throat	Ribose- and trehalose-fermented	
S. anginosus	ACGF and untypable	Beta (alpha or non-hemolytic gamma)	Throat, colon, female genital tract	Bacitracin-resistant, PYR-negative; minute colony variants of other groups	
Enterococcus sp. (*S. faecalis* and other enterococci)	D	Non-hemolytic	Colon, female genital tract throat	Growth in 6.5% NaCl; PYR-positive	Urinary tract infections, endocarditis, suppurative infections
Non-enterococcal Group D species (*S. bovis*)	D	Non-hemolytic	Colon	No growth in 6.5% NaCl	Endocarditis (*S. sanguinis*); dental caries (*S. mutans*)
Viridans streptococci (many species)	Not typed	Alpha	Mouth, colon, female genital tract	Optochin-resistant, species classification on biochemical properties	Infective endocarditis

Griffith typing is used for further classification of *S. pyogenes* belonging to Lancefield's Group A. Based on the M proteins on the cell surface, they are subdivided into eighty M types (M1, M2, M3 and so on).

STREPTOCOCCUS PYOGENES [GROUP A STREPTOCOCCI (GAS)]

Streptococcus pyogenes or Group A streptococcus is a human pathogen that

produces a diverse array of diseases, ranging from self-limited conditions (pharyngitis, impetigo and erysipelas) to severe invasive infections (bacteremia, necrotising fasciitis, myositis and streptococcal toxic shock syndrome). Streptococcal pharyngitis or skin infections may have lasting sequelae as post-streptococcal rheumatic disease or glomerulonephritis respectively.

There are several structural components of *Streptococcus pyogenes*, which are responsible for its pathogenesis. They include cell wall-associated factors, enzymes and toxins. Many of these are antigenic in nature and evoke an immune response. The immune response may be protective, or at times, may evoke a response manifesting as post-streptococcal sequelae. This is due to some of the structural antigens bearing similarity with host cell antigens. The virulence factors are discussed below.

Virulence Factors

Cell wall-associated factors

Several antigens are associated with the cell wall of *Streptococcus pyogenes* (Fig. 21.4). They play a major role in the pathogenesis of streptococcal infection.

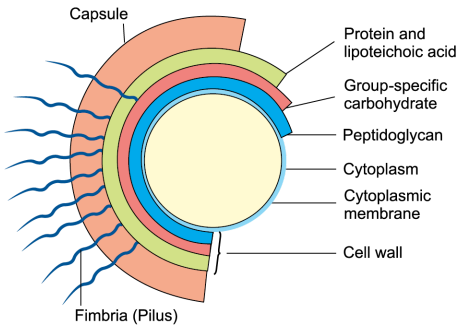


Fig. 21.4 Position of various antigens of *S. pyogenes*

1. ***Carbohydrate antigen*** On the basis of the C carbohydrate antigen, *S. pyogenes* is classified under Lancefield Group A. This antigen shows cross-reactivity with some human tissues (leads to post-streptococcal sequelae). Various methods are used to extract the antigen, e.g., Lancefield's hydrochloric acid extraction method, formamide **(Fuller's method)**, by an enzyme produced by *Streptomyces albus* (**Maxted's method**) or by autoclaving **(Rantz and Randall's method)**. The extract and the specific antisera are allowed

to react in capillary tubes. Precipitation occurs within five minutes at the interface between the extract and the homologous antiserum.

2. ***Protein antigen*** Several protein antigens have been identified in the outer part of the cell wall. *S. pyogenes* can be typed based on:

- **M proteins** (Griffith's M types) This is the most important protein used for typing as well as for virulence. It acts as a virulence factor by inhibiting phagocytosis and is antigenic. About 80 M protein types have been recognised. Rheumatogenic M types are 1, 3, 5, 6, 14, 18, 19, and 24. M types associated with pyoderma are 2, 49, 57, 59, 60, and 61.
- **T proteins** The T protein is an acid-labile, trypsin-resistant antigen present in many serotypes of *S. pyogenes*. It is demonstrated by the agglutination test using trypsin-treated whole streptococci.
- **R proteins** Some types of *S. pyogenes* (2, 3, 28 and 48) and some strains of groups B, C and G contain a third antigen, the R protein.
- A non-type-specific protein, associated with the M protein, known as the **M-associated protein** (MAP), has been identified.

3. ***Pili (fimbria)*** These hair-like structures project through the capsule of group A streptococci (GAS). The pili consist partly of M proteins and are covered with lipoteichoic acid which is important in the attachment of streptococci to epithelial cells.

Toxins

1. Hemolysins

Streptococci produce two hemolysins, streptolysin 'O' and 'S'. Streptolysin is produced by Group A (Groups C and G may also produce them).

- **Streptolysin O** is antigenic; anti-streptolysin O appears following streptococcal infection. Estimation of this antibody (**ASO titre**) is a standard serological procedure for the diagnosis of past infection with *S. pyogenes*. ASO test is done by the method of

latex agglutination. **An ASO titre in excess of 200 units is considered significant and suggests either recent or recurrent infection with streptococci**. Streptolysin O resembles the oxygen-labile hemolysins of *C. perfringens, C. tetani* and *S. pneumoniae.*

- **Streptolysin S** is an oxygen-stable hemolysin and is responsible for the hemolysis seen around streptococcal colonies on the surface of blood agar plates. It is called streptolysin S as it is soluble in serum. Though it is a protein, it is not antigenic. Convalescent sera do not neutralise streptolysin S activity. It is inhibited non-specifically by serum lipoproteins.

2. Streptococcal pyrogenic exotoxin (SPE)

SPE is also called erythrogenic **(or Dick or scarlatinal)** toxin. It is only of historical interest now.

Types of SPEs Three types of SPEs—**A, B** and **C**—have been identified. Types A and C are coded for by bacteriophage genes, while type B gene is chromosomal.

Superantigens
Like staphylococcal enterotoxins and the TSS toxin, SPEs are also superantigens. They are T cell mitogens that induce a massive release of inflammatory cytokines, causing tissue damage, fever and shock.

3. Streptokinase (fibrinolysin)

This toxin promotes the lysis of human fibrin clots by activating a plasma precursor (plasminogen), thereby causing the spread of infection. It is an **antigenic** protein, and neutralising antibodies appear in convalescent sera. Antistreptokinase antibodies provide retrospective evidence of streptococcal infection. **Streptokinase is given intravenously for the treatment of early myocardial infarction and other thromboembolic disorders**.

4. Deoxyribonucleases (streptodornase, DNase)

These cause depolymerisation of DNA and help to liquefy the thick pus in streptococcal exudate. A preparation containing streptokinase and streptodornase is available for liquefying the thick exudate in pyemia.

Types of DNases Four antigenically distinct DNases, **A, B, C** and **D**, have been recognised, of which type B is the most antigenic in human beings. **Anti-DNase B antibody** helps in the retrospective diagnosis of *S. pyogenes* skin infections, where ASO titres may be low.

5. Nicotinamide adenine dinucleotidase (NADase)

The biological significance of NADase is not known, though it is believed to be leucotoxic.

6. Hyaluronidase

This enzyme breaks down the hyaluronic acid of the tissues, favouring the spread of infection along the intercellular spaces. The enzyme is antigenic and specific antibodies appear in convalescent sera. Strains that form hyaluronidase in large quantities (M types 4 and 22) are non-capsulated.

7. Serum opacity factor (SOF)

Some M types of *S. pyogenes* produce a lipoproteinase which results in opacity in horse serum. This is a virulence determinant of the organism.

8. Other enzymes

Many strains also produce **proteinase, phosphatase, esterase, amylase, *N*-acetyl glucosaminidase, neuraminidase** and other toxins or enzymes. Their role in pathogenesis is unclear.

Pathogenesis and Clinical Findings

Streptococcal infections may be localised, diffuse (invasive) or may manifest as a sequelae to the original infection.

A. Localised infections

i) ***Streptococcal sore throat*** This is the most common streptococcal disease. It may be localised as tonsillitis or may involve the pharynx more diffusely **(pharyngitis**) as described (*Clinical case* 1). Virulent group A streptococci adhere to the pharyngeal epithelium by means of the lipoteichoic acid covering the surface pili. The glycoprotein fibronectin on the epithelial cells serves as the receptor to the lipoteichoic acid ligand. Tonsillitis is more common in older children and adults than in younger children, who commonly develop diffuse

pharyngitis. From the throat, streptococci may spread to the surrounding tissues, leading to suppurative complications such as otitis media, mastoiditis, quinsy, Ludwig's angina and suppurative adenitis.

Clinical Case 1

A seven-year-old girl presented with severe sore throat and fever up to 39°C for three days, with difficulty in swallowing. On examination, she had bilateral tender submandibular lymphadenopathy, enlarged tonsils and pharyngeal exudates. A culture of the throat swab was performed and, pending results, she was started on oral penicillin. The culture after 24 hours grew beta-hemolytic colonies on blood agar, which were gram-positive cocci in short chains, sensitive to penicillin. Diagnosis was confirmed as streptococcal pharyngitis.

ii) ***Skin infections*** Impetigo and pyoderma (Fig. 21.5) manifest as localised infections with induration tenderness and pus point. Impetigo and streptococcal infection of scabies lesions are the main causes of acute glomerulonephritis in children in the tropics. Antibody to DNase B and hyaluronidase are more useful in the retrospective diagnosis of pyoderma antecedent to acute glomerulonephritis.

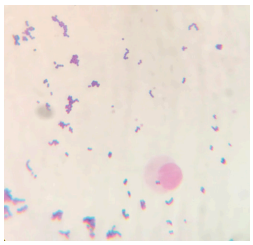


Fig. 21.5 Smear of pus from a pyoderma showing gram-positive cocci in chains, with polymorphs (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

iii) ***Erysipelas*** It is a rapidly progressing infection with brawny edema and a rapidly advancing margin.

iv) ***Cellulitis*** This refers to acute, rapidly spreading infection of the skin (Fig. 21.6a) and subcutaneous tissue with pain, tenderness and edema. Unlike erysipelas (Fig. 21.6b), the margin is diffuse between involved and uninvolved tissue. Systemic bacterial migration via the bloodstream can manifest as fever.

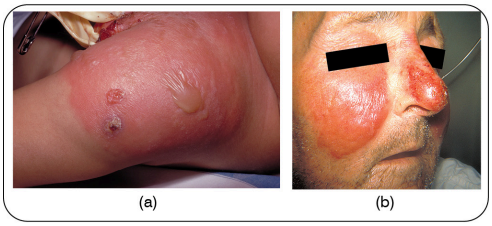


Fig. 21.6 (a) Cellulitis of a child's shoulder showing spreading erythema, swelling and accompanying areas of cutaneous purulency and (b) erysipelas affecting the face showing clear demarcation of the margin (*Source:* (a) Public Health Image Library, ID 4647 / Allen W. Mathies, MD / California Emergency Preparedness Office (Calif/EPO), Immunization Branch / CDC and (b) Public Health Image Library, ID 2874 / Dr. Thomas F. Sellers, Emory University / CDC)

v) ***Necrotising fasciitis*** Though this is more commonly caused by a mixed aerobic and anaerobic bacterial infection, some strains of *S. pyogenes* (more particularly **M types 1 and 3, which form pyrogenic exotoxin A**) alone may be responsible. In such cases, extensive necrosis of subcutaneous and muscular tissues and adjacent fascia (Fig. 21.7) is associated with a severe systemic illness—a toxic shock-like syndrome with disseminated intravascular coagulation and multiple system failure. *S. pyogenes* can be isolated from the affected site and rising titres of antistreptolysin and anti-DNase B demonstrated.

Fig. 21.7 Necrotising fasciitis after first debridement (*Source:* Wikimedia Commons)

Treatment Penicillin G, clindamycin or vancomycin are the drugs of choice in life-threatening infections.

B. Toxin-mediated infections

- ***Toxic shock syndrome (TSS)*** Soft tissue infections with some M types of *S. pyogenes* (1, 3, 12, 28) may sometimes cause a toxic shock syndrome resembling staphylococcal TSS. Streptococcal TSS and necrotising fasciitis occur in persons not immune to the infecting M types.

- ***Scarlet fever*** Pyrogenic exotoxins A–C causing scarlet fever may follow streptococcal pharyngitis or skin and soft tissue infections. Rashes on the trunk follow these infections.

> In the pre-antibiotic era, *S. pyogenes* was an important cause of death due to **puerperal sepsis**, with the infection usually being exogenous. The emphatic demonstration by Semmelweis in 1847 that hospital outbreaks of puerperal fever could be prevented by the simple measure of ensuring that those attending the labour wards wash their hands carefully remains a landmark in clinical microbiology.

Localisation is believed to be favoured by hypersensitivity due to prior contact.

C. Non-suppurative sequelae

S. pyogenes infections lead to two important non-suppurative post-streptococcal sequelae.

i) ***Acute rheumatic fever*** The pathogenesis of these complications is not clearly understood. The essential lesion in rheumatic fever is carditis, including connective tissue degeneration of the heart valves and inflammatory myocardial lesions characterised by **Aschoff nodules**. Typically, rheumatic fever follows persistent or repeated streptococcal throat infections with a strong antibody response to some **'rheumatogenic strains'** (*Clinical case 2*). The lesions are believed to be the result of hypersensitivity to some streptococcal component. It has also been suggested that an element of autoimmunity may be involved, and antigenic cross-reactions have been demonstrated between streptococci and heart tissues.

Clinical Case 2

A nine-year-old girl presented with a fever and sore throat. Group A streptococci were cultured from her throat. Three weeks later, she developed pain and tender swelling of both knees and ankles; a palpable effusion of the right knee was drained, which yielded straw-coloured fluid. Culture of the aspirate was negative. Simultaneously, the girl began experiencing shortness of breath. Physical examination and chest X-ray suggested mild congestive heart failure. The girl was diagnosed with acute rheumatic fever with reactive post-streptococcal arthritis. On follow-up after one year, a murmur was audible at the cardiac apex suggestive of rheumatic heart disease (RHD).

ii) ***Post-streptococcal glomerulonephritis*** Nephritis is caused by only a few **'nephritogenic' types**. In the tropics, skin infections are

perhaps more important in this respect than throat infections. Nephritis is usually a self-limited episode that resolves without any permanent damage. Its pathogenesis may be due to antigenic cross-reactions between the glomerular membrane antigen and cell membranes of nephritogenic streptococci. More often, it may be an immune complex disease (*Clinical case 3*).

Clinical Case 3

A seven-year-old boy developed a skin infection (pyoderma) for which his parents did not seek medical help. A week later, he passed dark-coloured urine. Four days later, he developed pitting edema in both ankles. On examination, the boy was found to have elevated blood pressure, 4+ proteinuria, red blood cell casts and elevated serum creatinine. He was diagnosed with acute post-streptococcal glomerulonephritis.

The important features of the two sequale are presented in Table 21.2.

Table 21.2 Comparison of rheumatic fever and post-streptococcal glomerulonephritis

Point of comparison	Acute rheumatic fever	Post-streptococcal glomerulonephritis
Primary site of infection	Throat	Mainly skin and pharyngitis
Prior sensitisation	Essential	Not necessary
Serotype of *S. pyogenes*	Any	Pyodermal types M 49, 53–55, 59–61; pharyngitic types 1, 4, 12 and 25
Immune response	Marked	Moderate
Complement level	Unaffected	Lowered
Genetic susceptibility	Present	Not known
Repeated attacks	Common	Absent
Penicillin prophylaxis	Essential	Not indicated
Course	Progressive or static	Spontaneous resolution
Prognosis	Variable	Good

iii) ***Pediatric autoimmune neuropsychiatric disorders associated with streptococcal (PANDAS) infections*** This was first noted in a series of case studies in the National Institute of Health, USA. A

hypothesis was made that there exists a subset of children with rapid onset of obsessive-compulsive disorder (OCD) or tic disorders in whom these symptoms are caused by Group A beta-hemolytic streptococcal (GABHS) infections. The proposed link between infection and these disorders is that an initial autoimmune reaction to a GABHS infection produces antibodies that interfere with basal ganglia function, causing symptom exacerbations. It has been proposed that this autoimmune response could result in a broad range of neuropsychiatric symptoms.

Epidemiology

S. pyogenes colonises the human upper respiratory tract—throat and nasopharynx—of patients and carriers (up to 20%).

Spread Transmission of infection is either by direct contact or through contaminated fingers, dust or fomites. Streptococcal infections of the respiratory tract are more frequent in children between 5–8 years of age than in children below the age of two years or in adults. Crowding is an important factor in the transmission of infection. Outbreaks of infection may occur in closed communities such as boarding schools or military camps.

Immunity It is type-specific and appears to be associated with the antibody to the M protein. Re-infections occur because of the multiplicity of serotypes.

Laboratory Diagnosis

In acute infections, diagnosis is established by culture, while in the non-suppurative complications, diagnosis is mainly based on the demonstration of antibodies.

1. **Specimen** Swabs or aspirates from the affected site are collected aseptically. Serum is collected for serology in rheumatic fever and glomerulonephritis.

Transport media If the sample cannot be transported immediately, Pike's transport medium is used.

2. **Microscopy** Presumptive diagnosis is by Gram-stained films from pus which show gram-positive cocci in chains. This is indicative of

streptococcal infection. **However, smears are of no value in infections of the throat or genitalia, where streptococci may form part of the resident flora.**

3. **Culture** The specimen is plated on 5% sheep **blood agar** and incubated at 37°C (range 22–42°C) anaerobically or under 5–10% CO_2 as hemolysis develops better under these conditions. Colonies on **blood agar**, after 24 hours, show small (0.5–1.0 mm), circular, semi-transparent colonies with clear hemolysis around them (Fig. 21.8).

Virulent strains, on fresh isolation from lesions, produce 'matt' (finely granular) colonies.

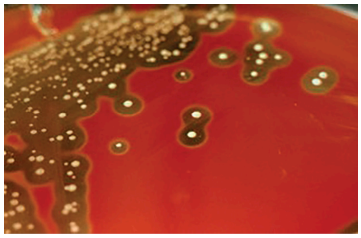


Fig. 21.8 Blood agar showing beta-hemolysis (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Avirulent strains form 'glossy' colonies.

Strains with well-marked capsules produce 'mucoid' colonies.

The organism can be stored for a long time in Robertson's cooked meat medium.

Selective media *S. pyogenes* is resistant to crystal violet. Crystal violet (1 mg/L), nalidixic acid (15 mg/L) and colistin sulphate (10 mg/L) added to blood agar provide a good selective medium for the isolation of streptococci, including pneumococci from specimen where mixed flora is expected. Another selective medium is the PNF medium (polymyxin, nalidixic acid and fusidic acid) which inhibits gram-negative bacteria and staphylococci.

4. **Identification**

- **Antigen detection** Rapid diagnostic test kits using specific antisera are available commercially for the detection of streptococcal Group A antigen from throat swabs. The tests can be completed in 1–4 hours and are nearly as specific as cultures, though less sensitive.

- **Bacitracin sensitivity** A wide zone of inhibition is seen around a 0.04 unit bacitracin with *S. pyogenes*, but not with other streptococci.
- **Biochemical reactions** Hydrolysis of pyrrolidonyl-beta-naphthylamide (PYR test) and failure to ferment ribose help differentiate *S. pyogenes* from other streptococci.

5. **Serology** In rheumatic fever and glomerulonephritis, a retrospective diagnosis of streptococcal infection may be established by demonstrating high levels of antibody to streptococcal toxins.

- **Antistreptolysin O (ASO)** The standard test is antistreptolysin O titration. ASO titres higher than 200 are indicative of prior streptococcal infection. The upper limit may vary with age, being higher in children than in adults. High levels are usually found in acute rheumatic fever but in glomerulonephritis, titres are often low.
- **Antideoxyribonuclease B (anti-DNase B)** This is an antibody to DNase. Titres higher than 300 are taken as significant. Anti-DNase B and antihyaluronidase tests are very useful for the retrospective diagnosis of streptococcal pyoderma, for which ASO is of much less value.
- **Streptozyme test** It is a passive slide hemagglutination test using erythrocytes sensitised with a crude preparation of extracellular antigens of streptococci. It is a convenient, sensitive and specific screening test. It is positive in nearly all types of streptococcal infections, whether of the throat or the skin.

6. **Typing**

- **Lancefield grouping** uses group-specific antisera; streptococci are typed in the laboratory.
- **MTR protein-based typing** of *S. pyogenes* is required only for epidemiological purposes and may be done by precipitation or agglutination techniques using specific antisera.

- **Emm typing** A non-serologic typing system for GAS is based on M-types (1–81) sequencing the 5' end of the M protein (emm) gene by a molecular-based typing system.

7. **Antimicrobial susceptibility** Streptococcus Group A are uniformly susceptible to penicillin. However, clinical failures are being reported following penicillin therapy. Hence**, monitoring MIC to penicillin is advisable in referral centres.** Strains resistant to cotrimoxazole and erythromycin have been reported and, therefore, antimicrobial susceptibility testing is required.

Treatment

All beta-hemolytic Group A streptococci are sensitive to **penicillin G,** and most are sensitive to **erythromycin.** In patients allergic to penicillin, erythromycin or **cephalexin** may be used. Tetracyclines and sulphonamides are not recommended. **Antimicrobial drugs have no effect on established glomerulonephritis and rheumatic fever**.

Bacitracin has been used for local application on skin lesions.

Prophylaxis

The indication for prophylaxis in streptococcal infections is directed at the prevention of rheumatic fever. This is achieved by the long-term administration of penicillin in children who have developed early signs of rheumatic fever. This prevents streptococcal re-infection and further damage to the heart. Antibiotic prophylaxis is not useful for glomerulonephritis as this complication follows a single streptococcal infection, and re-infections do not occur.

OTHER HEMOLYTIC STREPTOCOCCI

Besides *S. pyogenes*, streptococci belonging to Groups B, C, D, F, G and rarely H, K, O and R may also cause human infection. Of these, B, C and G are more common.

Group B Streptococci (GBS)

***Streptococcus agalactiae*,** the single most important bacterium of the GBS,

is an important human pathogen responsible for several infections. It is also an important pathogen of cattle, producing bovine mastitis. Nine capsular serotypes have been identified, antibodies to which confer type-specific protection.

Human infections include the following:

Neonatal infections Group B streptococcus has assumed great clinical importance as the single most common cause of neonatal meningitis in the West. Infection in the newborn is classified as:

i) **Early-onset type**, which occurs within a week of birth. The more common early-onset type presents as meningitis or septicemia, and is often fatal. Infection is acquired from the maternal vagina during birth.

ii) **Late-onset type**, which develops between the second and twelfth weeks of life. Infection is often acquired from the environment and presents as septicemia.

Other Group B infections in neonates include arthritis, osteomyelitis, conjunctivitis, respiratory infections, peritonitis, omphalitis and endocarditis.

Infections in adults This includes sepsis, skin and soft tissue infections, respiratory tract infections, and urinary tract infection in women. Human pathogenic Group B strains possess a polysaccharide capsule which appears to confer virulence. **Nine capsular serotypes have been identified**, antibodies to which confer type-specific protection.

Laboratory diagnosis

- On **blood agar**, the colonies show beta-hemolysis, as in the case of GAS.
- They hydrolyse hippurate.
- They are positive for **CAMP test** (Christie, Atkins and Munch–Peterson), in which an accentuated zone of hemolysis appears when *S. agalactiae* is inoculated perpendicular to a streak of *S. aureus* (*Staphylococcus plazens* streak produces beta lysin) on blood agar (Fig. 21.9).

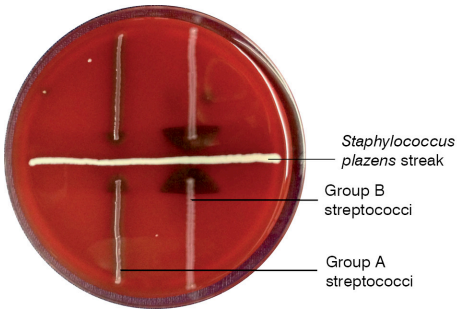


Fig. 21.9 CAMP test: Arrowhead shows hemolysis by *S. agalactiae*

Group C Streptococci

Streptococci of this group are predominantly animal pathogens. Group C strains isolated from human sources usually belong to the *S. equisimilis* species. It can cause upper respiratory infections, endocarditis, osteomyelitis, brain abscess, pneumonia and puerperal sepsis. Strains are often tolerant to penicillin. The addition of gentamicin is recommended in serious cases. *S. equisimilis* is the source of streptokinase used for thrombolytic therapy in patients.

Group F Streptococci

These grow poorly on blood agar unless incubated under CO_2. They have been called the **minute streptococci**. They can cause suppurative lesions. **Streptococcus MG** (belonging to Group F) is isolated from cases of primary atypical pneumonia. Demonstration of agglutinins to Streptococcus MG in the sera of patients was used as a diagnostic test for primary atypical pneumonia.

Group G Streptococci

These are commensals in the throats of human beings, monkeys and dogs. They may occasionally cause tonsillitis, endocarditis and urinary infections in human beings.

Group D Streptococci

Most of the members of this group are not human pathogens except *Streptococcus bovis*. It resides in the gastrointestinal tract. Members of this group are generally susceptible to penicillins. They may cause urinary infection or endocarditis.

Other Groups

Groups H and K sometimes cause infective endocarditis. Group O is isolated mainly from the healthy human throat and may cause acute tonsillitis and endocarditis. Group D has also been associated with bacteremia and infective endocarditis in adults. Recent studies have shown its association with colonic neoplasms and other gastrointestinal lesions.

Viridans Group

This group, formerly called *Streptococcus viridans*, is a miscellany of streptococci normally resident in the mouth and upper respiratory tract, and typically producing greening (alpha-lysis) on blood agar. Examples of organisms belonging to this group include *S. mitis, S. mutans, S. salivarius* and *S. sanguinis*.

Infections may cause **sub-acute bacterial endocarditis (SBE)**, *S. sanguinis* being most often responsible. Following tooth extraction or other dental procedures, they cause transient bacteremia and get implanted on damaged or prosthetic valves or in a congenitally diseased heart, and grow to form vegetation. **Prophylactic antibiotic cover is advisable in such persons before tooth extraction or similar procedures.**

OTHER STREPTOCOCCI

Streptococcus mutans is an alpha-hemolytic streptococcus which is part of the normal flora of the oral cavity. This gram-positive coccus is commonly found in the mouth, from where it can spread to cause dental caries or endocarditis in individuals with risk factors (dental extraction in people with damaged heart valves). The bacterium has a polysaccharide coat (glycocalyx) that allows it to stick to teeth and also to damaged heart valves; it can invade the bloodstream.

Nutritionally variant streptococci (NVS), also called nutritionally deficient streptococci, need pyridoxal or cysteine for their growth on blood agar. They are part of the normal flora but can be responsible for infective endocarditis, especially culture-negative infective endocarditis or brain abscess.

ENTEROCOCCI

The *Enterococcus* group includes *E. faecalis*, *E. faecium* and *E. durans*. Enterococci possess several features that are distinct from that of streptococci (Table 21.3). They typically appear as pairs of oval cocci, the cells in a pair arranged at an angle to each other (Fig. 21.10). They are usually non-hemolytic, though some strains may show alpha- or beta-hemolysis.

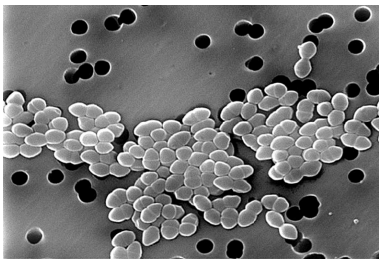


Fig. 21.10 *Enterococcus:* electron micrograph showing oval cells arranged in pairs (*Source:* Public Health Image Library, ID 266 / Janice Haney Carr / CDC)

Table 21.3 Distinguishing features of enterococci and streptococci

Characteristics	Enterococci	Streptococci
Shape	Oval cocci, sometimes at an angle to each other	Spherical
Length of chain	Short chains	Long chains
Growth on sheep blood agar	Generally non-hemolytic	Usually beta- or alpha-hemolytic
Growth on MacConkey medium	Tiny, magenta-pink colonies	Usually do not grow on MacConkey medium
Heat tolerance	Survive at 60°C for 30 minutes	Do not survive at this temperature
Growth characters	Grow in the presence of 40% bile, 6.5% sodium chloride, at pH 9.6, at 45°C and in 0.1% methylene blue milk	Do not grow under these conditions

E. faecalis is most often isolated from human sources. It can be identified by its ability to ferment mannitol, sucrose, sorbitol and esculin, and to grow on tellurite blood agar, producing black colonies. *Enterococcus faecium,* which is more resistant, can be differentiated by its ability to hydrolyse

arabinose.

Enterococci are frequently isolated from cases of urinary tract infection and wound infection. They may also cause endocarditis, infection of the biliary tract, septicemia and intra-abdominal abscess complicating diverticulitis and peritonitis.

Antimicrobial Resistance

Strains resistant to penicillin and other antibiotics occur frequently, so it is essential to perform antibiotic sensitivity for proper therapy. **Enterococci are intrinsically resistant to cephalosporins and offer low-level resistance to some aminoglycosides**; therefore, they require testing with high-level gentamicin discs in clinical laboratories.

> In penicillin-sensitive strains, synergism occurs with combination treatment with penicillin and aminoglycoside. However, if the strain shows high-level resistance to aminoglycosides, this synergism does not occur.

The choice of drug for infections due to such strains is vancomycin. Recently, **VRE** (**vancomycin-resistant enterococci**) have begun to emerge. The genes responsible for vancomycin resistance are *van A, B, C, D* and *E*. The mechanism is the alteration of the D-alanyl-D-alanine chain in the cell wall.

PNEUMOCOCCI

STREPTOCOCCUS PNEUMONIAE

Streptococcus pneumoniae is a gram-positive, lanceolate-shaped diplococcus (commonly referred to as pneumococci). Pneumococci are normal inhabitants of the human upper respiratory tract. They are the single most prevalent bacterial agents in pneumonia and in otitis media in children.

Morphology

S. pneumoniae are typically small (1 μm), slightly elongated diplococci, with one end broad or rounded and the other pointed, presenting a flame-shaped or lanceolate appearance. They are readily stained with aniline dyes

and are gram-positive (Fig. 21.11). The capsule may be demonstrated as a clear halo in India ink preparations or may be stained directly by special techniques. They are capsulated, the capsule enclosing each pair. The capsules are best seen in material taken directly from exudates and may be lost on repeated cultivation.

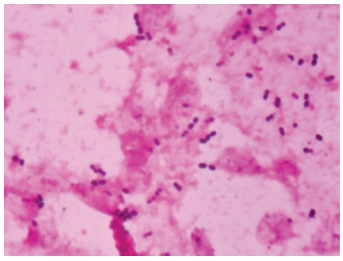


Fig. 21.11 Gram-stained smear of blood cultured in automated system containing lanceolate-shaped gram-positive cocci (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Antigenic Properties

1. ***Capsule*** The most important antigen of the *S. pneumoniae* is the type-specific capsular polysaccharide, also called the **specific soluble substance** (SSS). *S. pneumoniae* are classified based on the antigenic nature of the capsular polysaccharide, and now more than 90 different serotypes are recognised.

Serotyping

Currently, typing methods are based on Quellung reaction, latex agglutination and PCR-based methods.

- **Quellung reaction** This involves typing by the capsule swelling method. A suspension of *S. pneumoniae* is mixed on a slide with a drop of the type-specific antiserum and a loopful of methylene blue solution. In the presence of the homologous antiserum, the capsule becomes apparently swollen, sharply delineated and refractile (Fig. 21.12).
- **Latex agglutination** This involves typing by type-specific antiserum with the pneumococci.
- **PCR-based typing** Multilocus sequence typing (MLST), multilocus enzyme electrophoresis (MLEE) and DNA fingerprinting methods such as pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) are now being increasingly used for typing and characterising pneumococci. Typing by molecular methods is slowly replacing conventional methods.

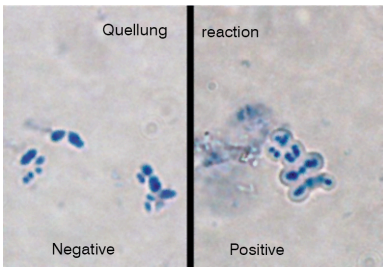


Fig. 21.12 Quellung reaction

2. ***Other antigens*** *S. pneumoniae* contains other antigens like the nucleoprotein deep inside the cell and a somatic '**C' carbohydrate antigen**, both of which are species-specific.

An abnormal acute-phase protein (beta-globulin), **C-reactive protein (CRP),** appears in the acute phase sera of pneumonia but disappears during convalescence. Its apparent antibody-like relationship to the 'C' antigen of *S. pneumoniae* is only incidental. It occurs in some other pathological conditions.

Smooth-rough variation
On repeated subculture, *S. pneumoniae* undergo **smooth-to-rough (S–R) variation**. In the R form, the colonies are rough and the cocci are non-capsulated, autoagglutinable and avirulent.

Toxins and Other Virulence Factors

The virulence of *S. pneumoniae* depends on the following factors:

- **Capsular polysaccharide**, because of its acidic and hydrophilic properties, protects the cocci from phagocytosis. Capsulated *S. pneumoniae* are not phagocytosed efficiently in fluid media or exudates. Non-capsulated strains are avirulent. **The antibody to the capsular polysaccharide affords protection against infection. This is the basis of vaccination.**
- **Pneumolysin**, a membrane-damaging toxin produced by *S. pneumoniae*, has cytotoxic and complement-activating properties and so, may be a virulence factor. It is immunogenic.
- **Pneumococcal autolysins**, by releasing bacterial components in infected tissues, may also contribute to virulence.

S. pneumoniae produces an oxygen-labile hemolysin and a leucocidin but these are weak and make no contribution to virulence.

Resistance

S. pneumoniae are delicate organisms and are readily destroyed by heat (thermal death point is 52°C for 15 minutes) and antiseptics. In culture, they die on prolonged incubation, perhaps due to an accumulation of toxic peroxides. Strains may be maintained on semisolid blood agar or by lyophilisation.

Pathogenesis

S. pneumoniae colonises the human nasopharynx and may cause infection of the middle ear, paranasal sinuses and lower respiratory tract by direct spread resulting in pneumonia. When the normal defences are compromised by viral infection, anesthesia, chilling or other factors, *S. pneumoniae* multiplies, penetrates the bronchial mucosa and spreads through the lung along the peribronchial tissues and lymphatics.

A. Invasive infections

Invasive infections due to *S. pneumoniae* occur through the bloodstream.

Clinical Case 4

A 60-year-old man was admitted with a history of high-grade fever, chills and rigors with mild chest pain and productive cough for the last two days. X-ray chest showed consolidation in the right lower lobe. The sputum smear observed after Gram-staining showed plenty of polymorphs with gram-positive, lanceolate-shaped diplococci. Sputum culture after 24 hours produced greenish colonies on blood agar, with bleaching on chocolate agar. Diagnosis was confirmed as pneumococcal pneumonia. Antibiotic susceptibility showed the organism to be sensitive to penicillin, to which he responded.

- **Lobar pneumonia** is commonly caused by *S. pneumoniae* (*Clinical case* 4). They also cause acute empyema. Bacteremia is common during the early stage of lobar pneumonia. Toxemia is due to diffusion of the capsular polysaccharide into blood and tissues. The drop in temperature by crisis and relief of symptoms coincide with the neutralisation of anticapsular antibodies.

Clinical Case 5

A five-year-old child was brought to the emergency department with a history of seizures following high-grade fever with 1–2 episodes of projectile vomiting. On examination, he had altered sensorium, and neck rigidity, with a positive Kernig's sign. A lumbar puncture after fundus examination was done for CSF cytology and biochemical examination. It showed

increased polymorphs with low glucose and raised proteins. Gram smear showed the presence of gram-positive diplococci. Culture was positive for alpha-hemolytic colonies on sheep blood agar with typical 'draughtsman colonies' that were optochin-sensitive, suggestive of S. pneumoniae. The patient was started on ceftriaxone and responded to treatment.

- **Bronchopneumonia** is almost always a secondary infection. This may be caused by any serotype of *S. pneumoniae*. The damage to the respiratory epithelium and excessive bronchial secretions caused by the primary infection facilitate the invasion of *S. pneumoniae* along the bronchial tree. Bronchopneumonia is frequently a terminal event in aged and debilitated patients. *S. pneumoniae* is commonly associated with acute exacerbations in **chronic bronchitis** and **tracheobronchitis.** The copious respiratory secretions in chronic bronchitis aid pneumococcal invasion. Another bacterium commonly associated with this condition is *Haemophilus influenzae*.
- **Meningitis** is the most serious of pneumococcal infections. It is usually secondary to other pneumococcal infections such as pneumonia, otitis media, sinusitis or conjunctivitis but in a proportion of cases, foci of infection may not be demonstrable. Pneumococcal meningitis occurs at all ages. Untreated cases are almost invariably fatal. Even with antibiotic therapy, the case fatality rate is about 25 per cent (*Clinical case* 5). Infection of the meninges may also occur, by contiguity or through blood.
- **Pneumococcal bacteremia** may also lead to distant infections in the heart, peritoneum or joints. Infection is commonly endogenous but exogenous infection may also occur, especially with highly virulent strains.

B. Suppurative lesions

S. pneumoniae also produce localised suppurative lesions in other parts of the body—otitis media, sinusitis, pericarditis, conjunctivitis, suppurative arthritis and peritonitis. It is also responsible for ocular infections like keratitis and dacryocystitis.

Epidemiology

The source of human infection is the respiratory tract of carriers and, less often, of patients. *S. pneumoniae* occurs in the throats of approximately half the population sampled at any time. It is transmitted by contaminated droplets or droplet nuclei. Dissemination is facilitated by crowding.

Serotypes associated with invasive infections

The case fatality rates of pneumonia may vary according to the virulence of the infecting serotype.

- Type 3 is the most virulent.
- Serotypes associated with invasive infections are 6, 14, 19F and 23F.
- In adults, types 1–8 are responsible for about 75 per cent of cases of pneumococcal pneumonia and bacteremia.
- In children, types 6, 9V, 14, 19 and 23 are frequent causes of meningitis.

Laboratory Diagnosis

The clinical diagnosis of suspected pneumococcal infections have to be confirmed by laboratory tests.

1. **Specimen** Sputum, CSF, blood and aspirated fluid are collected for direct demonstration, culture and antigen detection.

2. **Microscopy** In the acute phase of lobar pneumonia, the rusty sputum contains *S. pneumoniae* in large numbers, with hardly any other kind of bacterium. They are demonstrated by Gram stain (Fig. 21.13a). In acute otitis media, *S. pneumoniae* may be demonstrated in the fluid aspirated from the middle ear. In meningitis, presumptive diagnosis may be made from Gram-stained films of CSF (Fig. 21.13b). Gram-positive diplococci can be seen both inside the polymorphs (which are often scanty) and extracellularly.

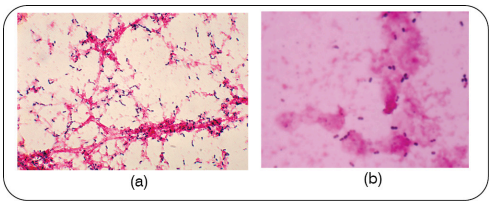


Fig. 21.13 Gram-positive diplococci with polymorphs: (a) in smear of sputum and (b) in CSF (*Source:* (a) Public Health Image Library, ID 2896 / Dr. Mike Miller / CDC) (b) Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

3. **Culture** In the acute stage of bacteremia, meningitis, pneumonia and other localised infections, the organism may be isolated from blood (in automated blood culture systems, or conventional brain heart infusion

broth), CSF, sputum and body fluids respectively.

S. pneumoniae have complex growth requirements and grow only in enriched media such as sheep blood agar and chocolate agar. They are aerobes and facultative anaerobes, the optimum temperature being 37°C (range 25–42°C) and pH 7.8 (range 6.5–8.3). Growth is improved by 5–10% CO_2. After incubation for 18 hours, the colonies that appear on blood agar are small (0.5–1 mm), dome-shaped and glistening, with an area of green discolouration (alpha-hemolysis) around them resembling colonies of viridans streptococci. They subsequently become flat with raised edges, central umbonation and concentric rings (draughtsman or carrom coin appearance; Fig. 21.14). On chocolate agar, they produce bleaching or light colour around the colony.

The cocci readily undergo autolysis in cultures due to the activity of the intracellular enzyme, autolysin. Autolysis is enhanced by bile salts.

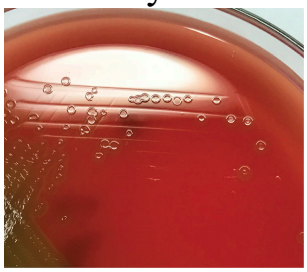


Fig. 21.14 Draughtsman or carrom coin appearance of pneumococcal colonies on sheep blood agar (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

4. **Biochemical reactions** Fermentation of inulin is a useful test for differentiating them from alpha- hemolytic streptococci, as the latter do not ferment it (Table 21.4).

Table 21.4 Differentiation between *S. pneumoniae* and Viridans streptococci

	S. pneumoniae	**Viridans streptococci**
Morphology	Capsulated, lanceolate, diplococci	Non-capsulated, oval or round cells in chains
Quellung test	Positive	Negative
Colonies on blood agar	Initially dome-shaped, later, 'draughtsman' colonies	Dome-shaped
Growth in liquid media	Uniform turbidity	Granular turbidity, powdery deposit

Bile solubility	Positive	Negative
Inulin fermentation	Positive	Negative
Optochin sensitivity	Positive	Negative
Intraperitoneal inoculation in mice	Fatal infection	Non-pathogenic

Bile solubility test The amidase on the cell wall is activated by surface-active agents such as bile or bile salts, resulting in the lysis of the organisms. If a few drops of 10% sodium deoxycholate solution are added to 1 mL of an overnight broth culture, the culture clears due to lysis of the cocci. Alternatively, if a loopful of 10% deoxycholate solution is placed on an *S. pneumoniae* colony on blood agar, the colony lyses within a few minutes. Bile solubility is a constant property of *S. pneumoniae* and hence is of diagnostic importance. The test should be carried out at neutral pH using deoxycholate and live young cells in saline suspension.

Optochin sensitivity *S. pneumoniae* is sensitive to optochin (ethyl hydrocuprein – 1/500,000), which differentiates it from streptococci. When a disc impregnated with optochin is applied to a plate of blood agar inoculated with *S. pneumoniae*, a wide zone of inhibition around the disc appears on blood agar after incubation (Fig. 21.15).

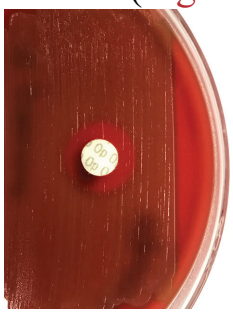


Fig. 21.15 *S. pneumoniae* colonies sensitive to optochin (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

5. **Antigen detection** Rapid diagnosis of pneumococcal meningitis can be done by antigen detection from CSF by latex agglutination test using **polyvalent antisera.**

Capsular polysaccharide can be demonstrated in blood, urine and cerebrospinal fluid by counterimmunoelectrophoresis. However, these tests are not very reliable.

6. **Biomarkers** **CRP** and **procalcitonin** are elevated in invasive

pneumococcal disease. The levels are monitored to determine prognosis and response to treatment.

7. **Molecular methods** PCR-based methods have much potential in cases where the patient has taken antibiotics.

Treatment

The antibiotic of choice is parenteral **penicillin** in severe cases and amoxycillin in milder ones, provided the infecting strain is penicillin-sensitive.

- **Penicillin resistance** Many penicillin-resistant strains are also resistant to other antibiotics like erythromycin and tetracycline. **The resistance may be intermediate (MIC 1 ∝g) or high (2 ∝g or more)**. The development of resistance is due to alteration in the **penicillin-binding protein** (PBP2a and PBP2x). Such strains are also resistant to multiple drugs. A drug-resistant *S. pneumoniae* (DRSP) strain originating in Spain has spread to most parts of the world, posing problems in treatment.
- **Third-generation cephalosporins** are indicated in penicillin-resistant isolates.
- **Vancomycin** is to be reserved for life-threatening illnesses with highly resistant strains.

Prevention of pneumococcal diseases (pneumococcal vaccines)

Immunity is type-specific and associated with antibodies to the capsular polysaccharide. The existence of some 90 serotypes makes a complete polyvalent vaccine impracticable. Protection would depend on whether the serotypes included in the vaccine are also prevalent in the community where the vaccine is used (Table 21.5).

Table 21.5 Some currently available pneumococcal vaccines

Current pneumococcal vaccines	Serotypes
23 Valent PPV-Polysaccharide Vaccine (Not recommended for children below 2 years)	Capsular antigens of the 23 most prevalent serotypes stated to give 80–90 per cent protection; used for adults

	and children above 5 years of age
7-valent conjugate vaccine targeting types (PCV7) Conjugated to CRM197 protein of *C. diphtheriae*	4, 6B, 9V, 14, 18C, 19F, 23F Can be used in children from two months to two years
10 valent pneumococcal conjugate vaccine (PHiD-CV) conjugated to NTHi protein	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5,7F
13-valent pneumococcal conjugate vaccine (PCV 13)	4, 6B, 9V, 14, 18C, 19F, 23F,1, 3, 5,6A, 7F, and 19A
15-valent PCV conjugated to CRM197	Serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F

Key points

- Bacteria belonging to the genus *Streptococcus* are gram-positive, oxidase- and catalase-negative and facultative anaerobes.
- Hemolytic activity has been used as a preliminary criterion for classifying some streptococci thus: alpha-hemolytic, beta-hemolytic or non-hemolytic (gamma-hemolytic).
- *Streptococcus pyogenes* is responsible for many human diseases, which are partly attributable to actual infection by the organisms (pharyngitis, impetigo and pyogenic infection) and immunological cross-reactions associated with streptococcal antigens (glomerulonephritis, rheumatic fever).
- Virulence factors include M protein and lipoteichoic acid, which help the bacteria to bind to cells, enzymes (hyaluronidase) which aid tissue spread, and toxins (erythrogenic toxin) which act as a superantigen. The isolate can be subtyped based on its M protein. Antibody to streptolysin can be estimated. Penicillin is often given for pharyngitis and skin infections to prevent more serious sequelae.
- *Streptococcus agalactiae* (Group B beta-hemolytic streptococci) can be found as normal flora in the vagina. It causes neonatal infections like neonatal septicemia, pneumonia and meningitis, and also infections of mothers during birth.
- *Streptococcus mutans* is an alpha-hemolytic streptococcus which is part of the normal flora of the oral cavity. It causes dental caries or endocarditis following dental extraction in people with damaged heart valves.
- *S. pneumoniae* occur as lanceolate-shaped, gram-positive diplococci in sputum, blood and CSF. They are alpha-hemolytic on blood agar. The bacterium is susceptible to optochin and to bile. Capsule swelling (the quellung phenomenon) occurs when the bacterium is mixed with a specific antibody.
- *Streptococcus pneumoniae* causes pneumococcal pneumonia (especially in the elderly and the very young) pneumococcal meningitis and pneumococcal keratitis and dacryocystitis.

- A major virulence factor is the polysaccharide capsule (> 90 types) that inhibits phagocytosis by polymorphonuclear leucocytes in blood, leading to invasion of the bloodstream and cerebrospinal fluid.
- Protection is by vaccines, namely 23 valent PPV, PCV7, 10 valent, 13 valent and 15 valent conjugate vaccines.
- Penicillin and cephalosporins are used to treat pneumococcal infections. Resistance to beta-lactams (penicillins, cephalosporins) is on the rise.
- Enterococci are part of the normal commensal flora of the gastrointestinal tract. *Enterococcus faecalis* is the commonest clinical isolate. Infection and disease are usually hospital-acquired and include urinary tract infections, abdominal and pelvic wound infections and bacteremia. Vancomycin-resistant enterococci are increasingly being isolated.
- Nutritionally-deficient streptococci or NVS produce infective endocarditis or brain abscess. They require pyridoxal for growth.

Questions

Essays

1. Classify streptococci and describe the pathogenesis of *Streptococcus pyogenes.*
2. Enumerate the virulence factors of Gr A streptococci and explain the laboratory diagnosis of streptococcal sore throat.
3. Draw a diagram of the cell wall of *Streptococcus pyogenes.*Explain the role of cell wall antigens in classification and virulence.
4. Describe the culture and identification of *Streptococcus pneumoniae*.
5. Describe the antigenic properties of the capsule and methods of typing.

Short Answers

1. Names of the viridans streptococci and the diseases caused by them
2. Diseases caused by streptococci
3. Post-streptococcal complications (non-suppurative complications)
4. Quellung reaction
5. Virulence factors of *S. pneumoniae*
6. Vaccines against *S. pneumoniae*
7. Infections produced by enterococci

CHAPTER 22

Neisseria and Moraxella

Chapter navigation

- *Neisseria meningitidis*
- *Neisseria gonorrhoeae*
- Non-gonococcal urethritis
- *Moraxella*

The genus *Neisseria* consists of gram-negative cocci, often arranged in pairs (diplococci). They are aerobic, non-sporulating, non-motile, oxidase-positive cocci. The genus contains two important human pathogens, *Neisseria meningitidis* and *Neisseria gonorrhoeae* while others in the genus are normal commensals rarely causing infection. They include *N. lactamica*, *N. flavescens*,
N. subflava and *N. sicca,* which occur as commensals in the mouth or upper respiratory tract. *Moraxella catarrhalis,* which is also found in the nasopharynx, can cause respiratory tract infections in immunocompromised hosts.

NEISSERIA MENINGITIDIS

Neisseria meningitidis (meningococcus) was first described and isolated in 1887 by Weichselbaum from the spinal fluid of a patient. Meningococci have been associated with several outbreaks of meningitis in both young children and adults.

Morphology

Meningococci are gram-negative, oval or spherical cocci, 0.6–0.8 ∝m in size, arranged typically in pairs, with the adjacent sides flattened (Fig. 22.1). Considerable variation occurs in size, shape and staining properties, especially in older cultures, due to autolysis. In smears from lesions, the

cocci are more regular and generally intracellular. They are non-motile. Most fresh isolates are capsulated.

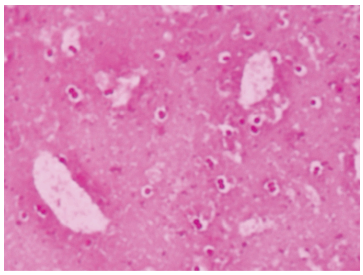


Fig. 22.1 *N. meningitidis* in cerebrospinal fluid; intracellular (within a polymorph) gram-negative diplococci with large number of polymorphs (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Clinical Case 1

A seven-year-old boy presented to the emergency department with high-grade fever and headache for two days and a single episode of convulsion and projectile vomiting the previous day. On examination, he was disoriented and his neck was found to be rigid; Kernig's sign was positive. Following funduscopic examination, a lumbar puncture was carried out, along with complete blood count and serum biochemistry. Cytology showed polymorphs at 1,000/mm^3, protein 250 mg/dL and glucose 20 mg/dL. Blood glucose levels were normal. On Gram stain, gram-negative cocci were seen in pairs, most of them intracellular with plenty of polymorphs. The latex agglutination test was positive for N. meningitidis antigen, confirming the diagnosis of meningitis. CSF culture was sent to the laboratory for isolation of the organism and AST. Empirically, ceftriaxone 100 mg/kg IV was started. Vaccination was advised for the patient's siblings along with ciprofloxacin chemoprophylaxis.

Antigenic Properties and Classification

Serogroups

Based on their capsular polysaccharide antigens, meningococci are classified into 13 serogroups, of which **Groups A, B, C, X, Y and W-135** are the most important. Group A is usually associated with epidemics and Group C with localised outbreaks, while Group B causes both epidemics and outbreaks. **Groups 29-E, W-135 and Y also frequently cause meningitis**. Any serogroup may colonise the nasopharynx, but these six groups account for most cases of meningitis.

Serotypes Serogroups are further classified into serotypes and subtypes based on the outer membrane proteins. Twenty such serotypes have been identified.

Meningococci have four virulence factors:

1. IgA protease that helps in binding to mucous membrane of upper respiratory tract by cleaving local IgA

2. Factor H binding protein that binds to Factor H, which is a C3b inhibitor that impairs complement activation
3. Endotoxin, which causes fever and shock
4. Capsular polysaccharide that resists phagocytosis

Clinical Spectrum of Meningococcal Infections

Meningococcal disease can present as cerebrospinal meningitis and meningococcal septicemia. Meningococci are strict human parasites inhabiting the nasopharynx. Infection is usually asymptomatic. In some, local inflammation ensues with rhinitis and pharyngitis. Dissemination occurs only in a small proportion.

Meningitis

The cocci spread from the nasopharynx to the meninges by travelling directly along the perineural sheath of the olfactory nerve, through the cribriform plate to the subarachnoid space, or more often, through the bloodstream. On reaching the central nervous system, a suppurative lesion of the meninges is set up, involving the surface of the spinal cord as well as the base and cortex of the brain. A typical presentation of pyogenic meningitis is discussed in *Clinical case* 1. The cocci are invariably found in spinal fluid, especially intracellularly in leucocytes. Case fatality is variable but may be as high as 80 per cent in untreated cases. Survivors may have sequelae such as blindness and deafness. Some cases develop chronic or recurrent meningitis.

Meningococcemia

This presents as acute fever with chills, malaise and prostration. Typically, a **petechial rash** occurs early in the disease. **Meningococci may be isolated from the petechial lesions**. Metastatic involvement of the joints, ears, eyes, lungs and adrenals may occur. About 10 per cent develop pneumonia. A few develop **fulminant meningococcemia** (formerly called **Waterhouse–Friderichsen syndrome**) which is an overwhelming and usually fatal condition, characterised by high fever, shock, disseminated intravascular coagulation and multisystem failure. Rarely, chronic meningococcemia may be seen. **Meningococcal disease is favoured by deficiency of the terminal complement components (C5–C9)**. This is due to the endotoxin

lipopolysaccharide released by autolysis. The vascular endothelium is particularly sensitive to the endotoxin. All major inflammatory cascade systems, as well as cytokines and nitric oxide, are triggered and upregulated. In fulminant cases, adrenal hemorrhage and profound shock are present.

Epidemiology

Natural infection is limited to human beings; there is no animal reservoir. Human nasopharynx is the only reservoir of the meningococcus. Asymptomatic nasopharyngeal carriers rarely contract the illness but serve to infect their contacts. Transmission is essentially airborne by droplets or less often, by fomites. During inter-epidemic periods, the carrier rate is about 5–10 per cent. **An increase in carrier rate heralds the onset of an epidemic**. During epidemics, the carrier rates in closed communities may go up to 90 per cent. Meningitis is common in children between the ages of three months and five years and in young adult population with a male preponderance. Epidemics usually occur in semi-closed communities living in crowded conditions.

Outbreaks have occurred in several states in India with serogroup A as the most common cause of epidemic and endemic infections. Between 2014 and 2015, a total of 16,217 cases and 300 deaths due to meningitis were recorded in India. Globally, the prevalence of meningitis is highest in the 'meningitis belt of Africa', stretching from Ethiopia to Senegal. One of the largest outbreaks was in 1996 when 150,000 cases and 15,000 deaths were reported.

Laboratory Diagnosis

The primary agents causing pyogenic meningitis are *N. meningitidis*, *S. pneumoniae* and *Haemophilus influenzae*. In meningococcal meningitis, the cocci are present in large numbers in spinal fluid and, in the early stages, in the blood as well.

1. **Specimens** Cerebrospinal fluid, blood, nasopharyngeal swab (in carriers) and skin scrapings from petechial lesions are the specimens collected depending on the clinical presentation.

2. **Examination of CSF** CSF appears turbid, with a large number of pus

cells. If the sample is adequate, it is divided as follows:

- **One portion** is centrifuged, and Gram-stained smears are prepared from the deposit. Meningococci are often seen extracellularly but a few are present intracellularly in the polymorphs. The supernatant may be used to demonstrate antigen by latex agglutination using meningococcal antisera. Commercially available kits containing antisera of *S. pneumoniae*, *H. influenzae* type b and Group B streptococcus are available for rapid diagnosis. **Antigen detection is particularly useful in partially treated patients in whom smear and culture tests may be negative**.
- The **second portion** of the CSF is cultured on blood agar and chocolate agar plates and incubated at 35–36°C under 5–10% CO_2. Colonies appear after 18–24 hours and are identified by glistening, translucent morphology and biochemical reactions. It must be remembered that morphologically similar organisms such as *N. flavescens*, *N. flava* and *Acinetobacter* may also occasionally cause purulent meningitis. The isolated meningococcus is serogrouped, if required, by agglutination with the appropriate sera.
- The **third portion** of the CSF is subjected to tests for biochemical parameters.

3. **Blood culture** In meningococcemia and in early cases of meningitis, blood culture is often positive. Cultures should be incubated for 4–7 days. Meningococcal antigens can be found in the blood in active disease.

4. **Aspirate from petechial lesions** Meningococci may sometimes be demonstrated in aspirates from petechial lesions by microscopy and culture.

5. **Cultural characteristics** Meningococci have exacting growth requirements and do not grow on ordinary media. Growth occurs on media enriched with blood serum.

They are aerobes but growth is facilitated by 5–10% CO_2 and high humidity. The optimum temperature for growth is 35–36°C and optimum pH 7.4–7.6. Meningococci are very delicate organisms, being highly

susceptible to heat, desiccation, alterations in pH and to disinfectants.

- After incubation for 24 hours on **solid media,** the colonies are small (about 1 mm in diameter), translucent, round, convex, bluish-grey, with a smooth glistening surface and entire edges. Weak hemolysis occurs on **blood agar**. Smooth and rough types of colonies are found. Growth is poor in **liquid media**.
- Thayer–Martin medium, Mueller–Hinton starch casein hydrolysate, blood agar and chocolate agar are used to culture meningococci.
- **Selective media** Modified Thayer–Martin medium (with vancomycin, colistin and nystatin) may be used as selective media to isolate meningococcus from carriers during an epidemic.

6. **Biochemical reactions** They are catalase- and oxidase-positive. The prompt oxidase reaction helps in the identification of *Neisseria* (both meningococcus and gonococcus) in mixed cultures.

- **Liquid oxidase test** This is used for the isolation of *Neisseria* from a mixed culture by plate method. When a freshly prepared 1% solution of oxidase reagent (tetramethyl paraphenylene diamine hydrochloride) is poured on a culture media with growth, the neisseriae colonies turn deep purple. Subcultures should be made immediately as the organism dies on prolonged exposure to the reagent.
- **Paper strip method** The test may be performed by rubbing a loop carrying a little of the growth on a strip of filter paper moistened with the oxidase reagent (**Kovac's method**). A deep purple colour appears immediately.

Glucose and maltose are utilised but not sucrose or lactose, producing acid but no gas (gonococci acidify glucose but not maltose). Acid formation by neisseriae is weak, being oxidative, and therefore, they are best tested on peptone serum sugars containing the sugar and indicator. Indole and hydrogen sulphide are not produced and nitrates are not reduced.

Rapid identification of suspected meningococcus isolates can be made by the VITEK systems, which also provide the MIC values in the antibiotic susceptibility panel.

7. **Molecular diagnosis** Group-specific diagnosis of infection can be made by the detection of meningococcal DNA sequence in CSF or blood by PCR amplification.

Treatment

Prompt treatment is essential to ensure recovery without sequelae. All strains were uniformly sensitive to penicillin and other antibiotics till a few years ago; however, resistant strains have emerged and become common in many countries.

Intravenous penicillin G is the treatment of choice. In persons who are hypersensitive to penicillin, alternative antibiotics are **ampicillin, ceftriaxone** or **cefotaxime**.

Ciprofloxacin or ceftriaxone may be used to decolonise a meningococcal carrier.

Prophylaxis

Penicillin cannot eradicate the carrier state. Ciprofloxacin is recommended for chemoprophylaxis. As infection rates are very high in the household or among close contacts of meningococcal patients, they should be provided with chemoprophylaxis.

Vaccines

1. **Polysaccharide vaccines**

- They are either bivalent (serogroups A and C), trivalent (A, C and W), or tetravalent (A, C, Y and W).
- They are not effective before 2 years of age.
- They offer 3-year protection but do not induce herd immunity.

2. **Conjugate vaccines**

- Monovalent C

- Monovalent A
- Tetravalent (serogroups A, C, Y and W)

Some countries like Cuba, New Zealand and Norway have outer membrane vesicle (OMV) vaccines, which are parenteral licensed vaccines against meningococcal B disease.

NEISSERIA GONORRHOEAE

Clinical Case 2

A 20-year-old man presented with purulent urethral discharge and dysuria. He had a history of unprotected sex with a commercial sex worker seven days before. On examination of a smear of the pus, intracellular gram-negative diplococci along with abundant polymorphs were found. The culture on Thayer–Martin medium was positive and a diagnosis of gonorrhea was made. The patient responded to the injection of ceftriaxone.

N. gonorrhoeae causes the venereal disease gonorrhea. The gonococcus was first described in gonorrheal pus by Neisser in 1879. Bumm, in 1885, cultured the coccus and proved its pathogenicity by inoculating human volunteers. Gonorrhea is a venereal disease that has been known since ancient times. The name gonorrhea (meaning flow of seed) was first employed by Galen in 130 AD (also known as the sin of Gomorrah).

Morphology

The organism appears as a kidney-shaped diplococcus. It is found predominantly within the polymorphs, some cells containing as many as a hundred cocci.

Gonococci possess pili on their surface. **Pili facilitate adhesion of the cocci to mucosal surfaces and promote virulence by inhibiting phagocytosis**. Piliated gonococci agglutinate human red blood cells but not those of other mammals. The hemagglutination is not inhibited by mannose.

Four types of colonial morphologies have been recognised based on the presence of pili: T1 to T4. Types 1 and 2 form small brown colonies. The cocci are piliated, autoagglutinable and virulent. Types 3 and 4 form larger, granular, non-pigmented colonies; they are non-piliated, form smooth suspensions and are avirulent. Fresh isolates from **acute cases of gonorrhea generally form T1 and T2** colonies. On serial subculture, they

switch to T3 or T4 colonial morphology. T1 and T2 types are also known as P^+ and P^{++} respectively, while T3 and T4 are known as P^-.

Antigenic Properties

Gonococci are antigenically heterogeneous. They are capable of changing their surface structures in vitro. They probably do so in vivo as well, to avoid host defence. The **surface structures** include the following:

- **Pili** These are hair-like structures, several micrometres long, and act as virulence factors by promoting attachment to host cells and inhibiting phagocytosis. Pili are composed of repeating peptide subunits (pilins). Pili undergo antigenic and phase variation.
- **Outer membrane proteins** Protein I is the major constituent and shows antigenic diversity, which helps in typing gonococcal strains. Protein I of a single strain is antigenically constant, though it shows considerable heterogeneity among different strains. It has two types, IA and IB. Any one strain carries only IA or IB but not both. Using monoclonal antibodies to protein I epitopes, gonococci can be classified into several **serovars, A1–24** and **B1–32.**
    - Proteins I and III act as ligands, attaching the coccus to the host cells. They also form transmembrane channels (porins) which play a role in the exchange of molecules across the outer membrane. Protein II is related to the opacity of the gonococcal colonies and so is called the **'opacity-associated' (OPA) outer membrane protein**. The outer membrane also contains **endotoxins,** which may be responsible for the toxicity in gonococcal infections.
    - These are **lipooligosaccharides** unlike the lipopolysaccharides of enterobacteriaceae.
- Many other proteins with poorly defined roles in pathogenicity have been described. Both gonococci and meningococci produce **IgA1 protease** that splits and inactivates IgA.

Resistance

The gonococcus is readily killed by heat, drying and antiseptics. It is a strict parasite and dies in 1–2 hours in exudates outside the body. In cultures, the coccus dies in 3–4 days but survives in slant cultures at 35°C if kept in sterile paraffin oil. Cultures may be preserved for years if frozen quickly and stored at –70°C.

Two transmissible plasmids contain genes that code for beta-lactamase which causes resistance to penicillin.

Clinical Spectrum of Gonococcal Infections

In the acute stage, diagnosis can be established readily but chronic cases sometimes present great difficulties.

Spread **The disease is acquired by sexual contact**. The first step in infection is adhesion of gonococci to the urethra or other mucosal surfaces. Pili are involved in this adhesion. The **incubation period** is 2–8 days.

- **In men**, the disease starts as acute urethritis with a mucopurulent discharge containing gonococci in large numbers (*Clinical case 2*). The infection extends along the urethra to the prostate, seminal vesicles and epididymis. Chronic urethritis may lead to stricture formation. The infection may spread to the periurethral tissues, causing **abscesses and multiple discharging sinuses** (**watercan perineum**).
- **In women, the initial infection involves the urethra and cervix uteri**. The vaginal mucosa is not usually affected in adults because the **stratified squamous epithelium is resistant to infection by the cocci and also because of the acidic pH of vaginal secretions**.

Infections in the urogenital tract are:

- **Vulvovaginitis** occurring in prepubertal girls
- **Pelvic inflammatory disease** (PID)
- **Salpingitis**, which may lead to sterility

- Rarely, peritonitis may develop with perihepatic inflammation (**Fitz-Hugh–Curtis syndrome)**; clinical disease is, as a rule, less severe in women, many of whom may carry gonococci in the cervix without any symptoms (asymptomatic carriage)
- **Disseminated gonococcal infection (DGI),** which is a severe form of systemic illness
- **Proctitis**, which occurs in both sexes; it may develop by direct contiguous spread in women but in men, it is usually the result of anal sex
- **Conjunctivitis,** which usually occurs due to autoinoculation by the patient's fingers
- **Blood invasion,** which may occur from the primary site of infection and may lead to metastatic lesions such as arthritis, ulcerative endocarditis and, very rarely, meningitis; occasional cases of **pyemia** have been reported
- **Ophthalmia neonatorum**, a non-venereal infection, is gonococcal ophthalmia in the newborn caused by direct infection during passage through the birth canal; once very common, this has been controlled by the practice of instilling 1% silver nitrate solution into the eyes of all newborn babies (**Crede's method**)
- **Gonococcal bacteremia,** leading to skin lesions, especially hemorrhagic papules and pustules on the hands, forearm, feet and legs, and to tenosynovitis and suppurative arthritis, usually of the knees, ankles and wrists

Epidemiology

Gonorrhea is an exclusively human disease. The existence of **asymptomatic carriage in women makes them a reservoi**r, serving to perpetuate infection among their male contacts.

The recent increase in antimicrobial resistance along with increased prevalence, has affected the treatment and control of infection.

Laboratory Diagnosis

1. **Specimen** Discharge or urethral swab and endocervical swab are the specimens collected for examination. The meatus is cleaned with a gauze

soaked in saline and a sample of the discharge collected with a platinum loop for culture, or directly on slide for smears. In women, besides the urethral discharge, cervical swabs should also be collected. This should be done carefully, using a speculum. High vaginal swabs are not satisfactory. In chronic infections, there may not be any urethral discharge. The 'morning drop' of secretion may be examined or some exudate may be obtained after prostatic massage. It may also be possible to demonstrate gonococci in the centrifuged deposits of urine in cases where no urethral discharge is available.

2. **Microscopy** Demonstration of intracellular, gram-negative diplococci in stained smears (Fig. 22.2) provides presumptive evidence of gonorrhea in men. It has to be emphasised that diagnosis of gonorrhea by smear examination is unreliable in women as some of the normal genital flora have essentially similar morphology. The use of fluorescent antibody techniques for the identification of gonococci in smears has increased the sensitivity and specificity of diagnosis by microscopy.

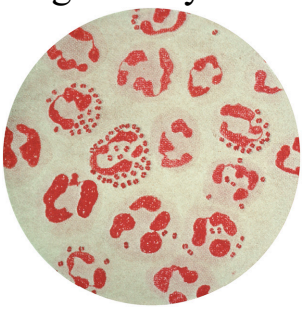


Fig. 22.2 Gonococci in urethral pus—gram-negative diplococci Source:Public Health Image Library, ID 2108 / CDC)

3. **Culture** For culture, specimens should be inoculated immediately after collection. If this is not possible, specimens should be collected with charcoal-impregnated swabs and sent to the laboratory in **Stuart's transport medium**. In acute gonorrhea, cultures can be obtained readily on chocolate agar or **Thayer–Martin medium** (modified Thayer–Martin medium with vancomycin, colistin and nystatin) at 35–36°C under 5–10% CO_2. Growth is identified by morphology and biochemical reactions. Gonococci are more difficult to grow than meningococci (Table 22.1). Colonies are small, round, translucent, convex or slightly umbonate, with a finely granular surface and lobate margins.

Table 22.1 Differential characteristics of *Neisseriae*

Species	Colonies	Growth on nutrient agar and at 22°C	Fermentation of Glu, Suc, Malt	Serological classification
N. meningitidis	Round, smooth, shiny, creamy consistency	Negative	Glu and Malt	Thirteen antigenic groups
N. gonorrhoeae	Same as above, but smaller and more opalescent	Negative	Glu only	Antigenically heterogeneous
N. catarrhalis (Branhamella catarrhalis) Resembles meningococcus but is pigmented yellow	Variable, smooth and translucent or adherent and opaque, not easily emulsifiable	Grows on nutrient agar and also at 22°C	Glu and Malt	Autoagglutinates

4. **Biochemical reactions** They acidify only glucose and not maltose in serum sugars.

Rapid carbohydrate utilisation test (RCUT) This is a rapid sugar fermentation test that is now recommended. It is based on the presence of pre-formed enzymes in the bacteria and is not dependent on the growth of bacteria in the sugar media. Though recommended, it is not used widely now as the more sensitive method of the VITEK system is available.

5. **Serological tests** No serological test has been found to be useful for routine diagnostic purposes.

6. **Molecular methods** PCR molecular methods have improved the sensitivity of diagnostic assay but are not cost-effective for routine use.

Treatment

Drug resistance
From 1957, strains with decreased susceptibility (MIC higher than 0.1 unit/mL) became common. From 1976, gonococci producing beta-lactamase (penicillinase) have appeared, rendering penicillin treatment ineffective. Gonococcal resistance to tetracycline first emerged in Asia during the 1970s. High levels of resistance to quinolones (e.g., ciprofloxacin) developed by the mid-2000s.

Penicillinase-producing *N. gonorrhoeae* (PPNG) have spread widely.

Latest data indicate increasing gonococcal resistance to, and treatment failures with, third-generation oral cephalosporins. Several of the gonococcal strains associated with failure of cephalosporin treatment have also demonstrated resistance to other antibiotics and have been classified as **multidrug-resistant gonococci (MDR)**. Following this, there has been an emergence of extensively drug-resistant (XDR) *Neisseria gonorrhoeae*. These include QRNG (quinolone-resistant NG), TRNG (tetracycline-resistant NG) and a group known as CMRNG (chromosomally-mediated resistant NG).

Recommended schedule

WHO recommends cefixime + azithromycin, and ceftriaxone + azithromycin combinations. As recommended by CDC (Centers for Disease Control and Prevention), since 2006, due to increase in ciprofloxacin resistance, the recommended treatment protocol is ceftriaxone 125 mg IM single dose, with 1 g azithromycin single dose or with doxycycline 100 mg twice a day for 7 days. The regime works well against gonococci and the frequently co-existing chlamydial infection.

Prophylaxis

Control of gonorrhea consists of early detection of cases, contact tracing, health education and other general measures. Clinical disease does not confer any immunity, and **vaccination has no place in prophylaxis.**

NON-GONOCOCCAL (NON-SPECIFIC) URETHRITIS

Along with an increase in the incidence of gonorrhea, there has also been an increase, in recent years, of cases of chronic urethritis where gonococci cannot be demonstrated. This has been called non-gonococcal or non-specific urethritis. In some of these, urethritis forms part of a syndrome consisting of conjunctivitis and arthritis in addition (**Reiter's syndrome**). Some of these cases may be due to gonococcal infection, the cocci persisting as L forms and hence undetectable by routine tests. The majority of such cases are, however, the result of infections of diverse origin (Table 22.2). The most important of these are *C. trachomatis, Ureaplasma urealyticum* and *Mycoplasma hominis*. Herpesvirus and cytomegalovirus may also account for some cases. Urethritis may also be caused by other

bacteria (e.g., *Gardnerella vaginalis, Acinetobacter lwoffii, A. calcoaceticus*), fungi (*Candida albicans*), protozoa (*Trichomonas vaginalis*), or even by mechanical or chemical irritation. As etiological diagnosis is seldom achieved, the management of this syndrome is difficult.

Table 22.2 Salient differences between gonococcal and non-gonococcal urethritis

	Gonococcal urethritis	**Non-gonococcal urethritis**
Causative agent	Bacteria *N. gonorrhoeae*	Bacteria, viruses, fungi and protozoa *C. trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma hominis*, herpesvirus and cytomegalovirus (*Candida albicans*), protozoa (*Trichomonas vaginalis*)
Transmission	Sexual	Sexual
Diagnosis	Microscopy and culture	*C. trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma hominis* by immunofluorescence and antigen detection Herpesvirus and cytomegalovirus by immunofluorescence, antigen detection and PCR *Candida albicans* by microscopy and culture *Trichomonas vaginalis* by wet mount examination by microscopy

COMMENSAL NEISSERIAE

Several species of neisseriae inhabit the normal respiratory tract. The characteristic features of some of the common species are listed in Table 22.1. Their pathogenic significance is uncertain though some of them (e.g., *N. flavescens, N. catarrhalis*) have been reported occasionally as having caused meningitis.

MORAXELLA

The genus *Moraxella* belongs to the family Moraxellaceae. They are gram-negative diplococci or coccobacilli. One of the common species ***Moraxella (Branhamella) catarrhalis*** was earlier classified as *N. catarrhalis*. Other species of *Moraxella* are *M. lactamica, M. bovis* and *M. oslonensis.*

Moraxella catarrhalis is an exclusively human opportunistic pathogen and is a common cause of otitis media in infants and children. The tendency of

the organism to colonise the upper respiratory tract is high in infants and children but decreases in adulthood. It can cause sinusitis, laryngitis, bronchopneumonia and meningitis. In a significant proportion of the elderly patients with chronic obstructive pulmonary disease and chronic bronchitis, it causes lower respiratory tract infections.

M. catarrhalis resembles commensal *Neisseria* species in culture. It is a gram-negative diplococcus, and is catalase- and oxidase-positive. These organisms are inert biochemically (asaccharolytic). Moraxella grow on ordinary media, unlike the pathogenic *Neisseria*.

Treatment

Most strains produce β-lactamase and are thus resistant to ampicillin and other penicillin group antibiotics. They are susceptible to other classes of oral antimicrobial agents.

Key points

- Bacteria belonging to the genus *Neisseria* are gram-negative cocci occurring in pairs. The two important pathogenic species are *Neisseria meningitidis* and *Neisseria gonorrhoeae*.
- Non-pathogenic species of *Neisseria* can occur as commensals.
- *Neisseria meningitidis* (meningococcus) is a common cause of meningitis. It can be life-threatening if untreated.
- *Neisseria gonorrhoeae* (gonococcus) causes gonorrhea, a sexually transmitted infection.
- *Neisseria meningitidis* and *Neisseria gonorrhoeae* are relatively difficult to grow (fastidious) and require enriched culture media and an atmosphere with high humidity and enhanced carbon dioxide concentration for isolation from specimens.
- *Moraxella catarrhalis* is an exclusively human opportunistic pathogen and is a common cause of otitis media in infants and children. In a significant proportion of the elderly patients, it causes lower respiratory tract infection.

Questions

Essays

1. Describe the pathogenesis and laboratory diagnosis of meningococcal meningitis.
2. Describe the mode of transmission and laboratory diagnosis of *N. gonorrhoeae*.

Short Answers

1. Four differences between *N. meningitidis* and *N. gonorrhoeae*
2. The vaccines to prevent meningococcal meningitis

3. Serotypes of *N. meningitidis*
4. Antibiotic resistance in *N. gonorrhoeae*

Part III: Bacteriology

Chapter 23

Corynebacterium

Chapter navigation

- *Corynebacterium diphtheriae*
- Diphtheria
- Diphtheroids

INTRODUCTION

Corynebacteria are gram-positive, non-sporing, non-acid-fast, non-motile rods with irregularly stained segments due to the presence of granules in some species. They frequently show club-shaped swellings—hence the name *Corynebacterium* (from *coryne*, meaning 'club').

The most important member of the genus is *C. diphtheriae,* the causative agent of diphtheria. The bacillus was first observed and described by Klebs (1883) and was first cultivated by Loeffler (1884); hence it is known as the Klebs–Loeffler bacillus or KLB. Roux and Yersin (1888) discovered the diphtheria exotoxin and established its pathogenic effect. The antitoxin was described by von Behring (1890).

CORYNEBACTERIUM DIPHTHERIAE

Clinical Case

A five-year-old child presented to the outpatient ward with a history of pain in the throat and difficulty in swallowing accompanied by low-grade fever for the past two days. He had enlarged cervical lymph nodes and his tonsillar pillars were covered with a grey-white discharge. His vaccination card revealed incomplete adherence to the immunisation schedule. Albert's stain of the throat swab smear showed rod-shaped bacteria, green in colour, with bluish-black granules (Fig. 23.1a). A presumptive diagnosis of diphtheria was made and the child was immediately started on penicillin–50 mg/kg (maximum 1.2 g) a day followed by passive immunisation with diphtheria antitoxin. Prophylactic erythromycin was prescribed to the child's siblings along with vaccination depending on the status.

Morphology

Corynebacterium diphtheriae is pleomorphic, measuring approximately 3–6 µm × 0.6–0.8 µm. Although gram-positive, they tend to be decolourised easily. They possess **polymetaphosphate granules** that serve as energy storage granules and are called **volutin** or **Babes–Ernst granules** or **metachromatic granules** (Fig. 23.1a). These give the bacilli a beaded appearance when stained with aniline dyes. The bacilli are arranged in characteristic pairs, palisades (parallel rows) or small groups. They often appear at angles to each other, resembling the letters V or L. This has been called the **Chinese letter** or **cuneiform** arrangement due to incomplete separation of the daughter cells after binary fission (Fig. 23.1b).

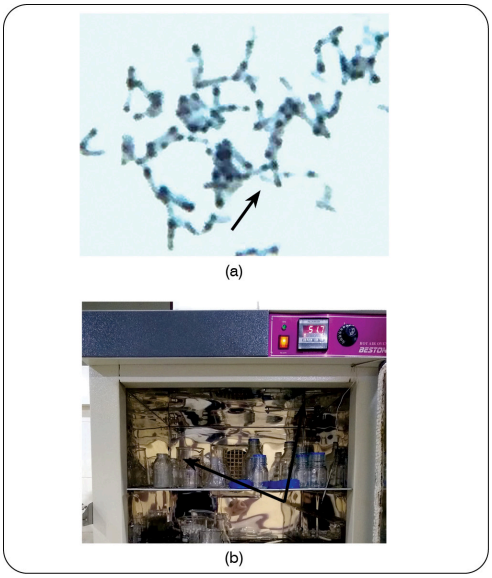


Fig. 23.1 *C. diphtheriae:* (a) metachromatic granules in Albert stain and (b) cuneiform arrangement (V and L shapes) (*Source:* (a) Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Antigenic Structure

C. diphtheriae have been typed as gravis, intermedius and mitis based on several characters. Gravis strains have been classified into 13 antigenic types, intermedius into 4 and mitis into 40 types. No connection has been established between type specificity and other characters.

Pathogenicity

Toxin

Virulent strains of **diphtheria bacilli produce a very powerful exotoxin** (Table 23.1). The pathogenic effects of the bacillus are due to the toxin. Almost all strains of gravis and intermedius (about 95–99 per cent) are toxigenic, while only about 80–85 per cent of mitis strains are toxigenic.

The standard strain almost universally used for toxin production is the **Park–Williams 8 strain.**

Table 23.1 Characteristics of diphtheria toxin

Lethal dose	0.1 ∝g/kg
Structure	2 subunits a) A—Active domain, responsible for action b) B—Binding domain, triggers entry into host cell
Host cell receptor	CD9 and HBEGF-like precursor
Mechanism	Entry is by attachment to receptor (endocytosis) Action—inhibits protein synthesis by inactivating EF2 (similar action demonstrated by exotoxin of *Pseudomonas aeruginosa*)

Properties

The diphtheria toxin is a protein that can be crystallised. It has a molecular weight of about 62,000. It is extremely potent and the lethal dose for a 250 g guinea pig is
0.0001 mg. It consists of two fragments, **A and B,** of MW 24,000 and 38,000 respectively. Both fragments are necessary for the toxic effect. When released by the bacterium, the toxin is inactive because the active site on fragment A is masked. Activation is probably accomplished by proteases present in infected tissues. Fragment A is the toxic enzyme. Fragment B (Table 23.1) is responsible for binding the toxin to the cells. The antibody to fragment B protects by preventing the binding of the toxin to the cells. The toxin is labile. Prolonged storage, incubation at 37°C for 4–6 weeks, treatment with 0.2–0.4% formalin or acid pH converts it to toxoid. **Toxoid is a toxin that has lost its toxicity but not its antigenicity.** It is capable of inducing antitoxin antibodies and hence is used as a vaccine candidate.

The **factors affecting toxin production** are as follows:

- The toxigenicity of the diphtheria bacillus depends on the intracellular presence of **corynephages (tox+),** which act as the

genetic determinant controlling toxin production.

- Non-toxigenic strains may be rendered toxigenic by infecting them with **beta** or some other phage. This is known as **lysogenic** or **phage conversion**. The toxigenicity remains only as long as the bacillus is lysogenic.
- Toxin production is also influenced by the concentration of **iron** in the medium. The optimum level of iron for toxin production is 0.1 mg/L, while a concentration of 0.5 mg/L inhibits the formation of toxin.

Mechanism of action

The diphtheria **toxin acts by inhibiting protein synthesis**. Fragment B helps in binding while fragment A inhibits polypeptide chain elongation in the presence of nicotinamide adenine dinucleotide (NAD) by **inactivating the elongation factor, EF-2.** It has a special affinity to certain tissues such as the myocardium, adrenals and nerve endings.

Clinical Features

The **incubation period** in diphtheria is commonly 3–4 days but may, on occasion, be as short as one day. In carriers, the incubation period may be very prolonged.

The **site** of infection may be:

- Pharyngeal (most common)
- Laryngeal
- Otitic
- Nasal
- Genital—vulval, vaginal or preputial
- Conjunctival
- Cutaneous

Pharyngeal diphtheria is the most common type and may vary from mild catarrhal inflammation to very widespread involvement (*Clinical case*).

Diphtheria may be classified based on **clinical severity** as:

a) **Malignant** or **hypertoxic,** in which there is severe toxemia with marked adenitis (bull neck). Death is due to circulatory failure. There is a high incidence of paralytic sequelae in those who recover.
b) **Septic**, which leads to ulceration, cellulitis and even gangrene around the pseudomembrane.
c) **Hemorrhagic**, which is characterised by bleeding from the edge of the membrane, epistaxis, conjunctival hemorrhage, purpura and generalised bleeding tendency.

The common complications are as follows:

- **Asphyxia** due to mechanical obstruction of the respiratory passage by the pseudomembrane, for which an emergency tracheostomy may become necessary.
- **Acute circulatory failure**, which may be peripheral or cardiac.
- **Post-diphtheritic paralysis**, which typically occurs in the third or fourth week of the disease; palatine and ciliary, but not papillary, paralysis is characteristic, and spontaneous recovery is the rule.
- **Toxemia,** when toxin is absorbed and produces toxic damage to the heart (myocarditis), kidney (tubular necrosis), liver and adrenal glands.
- **Local necrotic changes**, leading to fibrinous exudates; these, together with the disintegrating epithelial cells, leucocytes, erythrocytes and bacteria, constitute the pseudomembrane, which is characteristic of diphtheritic infection.
- **Mechanical**, caused by the membrane.

Non-toxigenic strains may cause infection even in immunised individuals, as immunity with the toxoid does not confer antibacterial immunity. Such infection is mild, though pseudomembrane formation may sometimes occur, leading to a fatal outcome.

Cutaneous diphtheria *Corynebacterium* infects the skin more often than the respiratory tract in the tropics. Toxigenic corynebacteria may persist in the skin for over three years. Cutaneous infections may stimulate natural immunity to diphtheria but can also lead to **pharyngeal diphtheria** in non-immune contacts. Cutaneous infections are usually secondary to pre-

existing skin lesions. Sometimes, **diphtheritic whitlow** or ulcer may occur. Cutaneous infections are commonly caused by non-toxigenic strains of *C. diphtheriae*.

Typing

Typing methods are used to determine the transcontinental spread.

Methods for the typing of *C. diphtheriae*

- **Biotyping** This is done based on biochemical tests, mostly using automated systems.
- **Ribotyping** This is presently considered to be the most useful method to type the strains of *C. diphtheriae*.
- **Molecular methods Pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD)** or **amplified fragment length polymorphism (AFLP)** are the other molecular methods used for typing.
- **Bacteriophage typing** About 15 bacteriophage types have been described. Types I and III are mitis, IV and VI intermedius, VII avirulent gravis and the remainder, virulent gravis. The phage types are apparently stable.

A system of bacteriocin (diphthericin) typing has also been described. Other methods of typing include bacterial polypeptide analysis, DNA restriction patterns and hybridisation with DNA probes. However, due to poor discriminatory power, this is not used any longer.

Laboratory Diagnosis

Laboratory confirmation of diphtheria is necessary for the initiation of control measures and for epidemiological purposes but not for the treatment of individual cases. **Specific treatment should be instituted immediately on suspicion of diphtheria without waiting for laboratory tests.**

1. **Specimen** Preferably, two dacron swabs from the lesions are collected from the lesion under vision, using a tongue depressor. The area under the visible membrane should be sampled.

2. **Microscopy** Smears are stained with methylene blue or one of the special stains.

- ***Neisser's* or *Albert's stain*** This stain will show the bacilli with metachromatic granules and in the typical cuneiform arrangement [Albert's stain may show delicate green bacilli with purple-blue metachromatic granules (Fig. 23.1a and b)]. The

granules are often situated at the poles of the bacilli and are called **polar bodies**.

- ***Gram stain*** The granules are more strongly gram-positive than the rest of the bacterial cell and appear slightly swollen, thus giving it a club shape (Fig. 23.2).

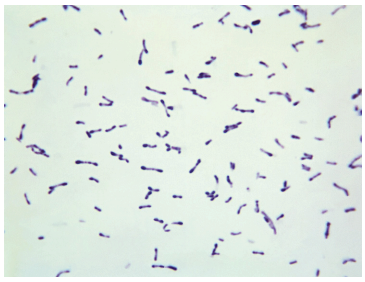


Fig. 23.2 Gram stain showing club-shaped bacilli (*Source:* Public Health Image Library, ID 12163 / Graham Heid / CDC)

- ***Loeffler's methylene blue*** The granules take up a bluish-purple colour and are, hence called **metachromatic granules.** However, the bacilli may not always be demonstrable in smears from the lesion; confident differentiation from some commensal corynebacteria normally found in the throat may also be difficult.
- ***Leishman stain*** This is done to rule out Vincent's spirochetes and fusiform bacilli responsible for Vincent's angina which clinically resembles diphtheria.
- ***Immunofluorescence*** Diphtheria bacilli may be identified in smears by **direct immunofluorescence test**. This is most specific as the smear stained with specific antibody is tagged with fluorescent dye.

3. **Isolation by culture** Enrichment with blood, serum or egg is necessary for good growth. The optimum temperature for growth is 37°C (range 15–40°C) and the optimum pH is 7.2. *C. diphtheriae* is an aerobe and a facultative anaerobe. The following specific media are used in the laboratory to isolate and identify the bacteria:

- ***Loeffler's serum slope*** Growth is rapid on Loeffler's serum slope but colonies are difficult to visualise. A smear is made after 6–8 hours of inoculation of the water of condensation in the test tube and stained with Albert's stain to demonstrate granules.
- ***Sheep blood agar*** This is required to differentiate colonies from streptococcal or staphylococcal pharyngitis, which may simulate diphtheria. Colonies are greyish and moist, showing variable hemolysis based on the biotype.
- ***Tellurite agar or tellurite blood agar*** Several modifications of tellurite blood agar have been utilised, such as **McLeod's** and **Hoyle's** media. Tellurite (0.04%) inhibits the growth of most other bacteria, acting as a selective agent. Diphtheria bacilli reduce tellurite to metallic tellurium, giving the colonies a grey or black colour (Fig. 23.3). **Plates are incubated for at least two days before being considered negative, as growth may sometimes be delayed.**

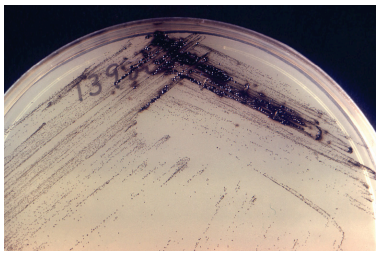


Fig. 23.3 Black colonies of corynebacteria on tellurite agar (*Source:* Public Health Image Library, ID 16561 / CDC)

Based on colonial morphology on the tellurite medium and other properties, *C. diphtheriae* is classified as mentioned earlier into the three biotypes—**gravis, intermedius** and **mitis** (Table 23.2).

Table 23.2 Characteristics of the three biotypes of *C. diphtheriae*

	Gravis	**Mitis**	**Intermedius**
Morphology	Short rods, uniformly stained, with few or no granules; some degree of pleomorphism, with tear-drop forms	Long, barred forms with clubbed ends; poor granulation, very pleomorphic	Long, curved, pleomorphic rods with prominent granules
Colony on tellurite blood	Greyish-black centre, 1–2 mm in size, crenation of	Small, around 1 mm in size, misty; dull	Variable size, shiny black colonies in 2–

agar in 18 hours	edge; later, flat colony with raised dark centre and crenated edge and radial striation: 'daisy head' colony	granular centre and glistening periphery with a ring near the edge: 'frog's egg' colony	3 days, later flat, with central elevation: 'poached egg' colony
Consistency of the colonies	Like 'cold butter'; brittle; moves as a whole on the plate, not easily picked out or emulsifiable	Intermediate between gravis and mitis	Soft, buttery, easily emulsifiable
Hemolysis and growth in broth	Variable Surface pellicle, granular deposit, little or no turbidity	Non-hemolytic Turbidity in 24 hours, clearing in 48 hours, with fine granular sediment	Usually hemolytic Diffuse turbidity with soft pellicle later
Glycogen and starch fermentation	Positive	Negative	Negative

4. **Biochemical reactions**

Hiss's serum sugars *C. diphtheriae* ferments glucose, galactose, maltose and dextrin with the production of acid without gas; it does not ferment lactose, mannitol or sucrose (although some virulent strains might).

5. **Demonstration of toxicity (virulence testing)** Any isolate of *C. diphtheriae* should be tested for toxigenicity for the bacteriological diagnosis to be complete.

In vivo animal tests are not recommended any more because they are prohibited by animal protection acts and also due to the availability of more specific in vitro tests.

In vitro tests to detect diphtheria toxin

- ***Elek's gel precipitation test*** This is the conventional filter paper strip method **to detect toxin production by clinical isolates** (Fig. 23.4). A modified test (recommended by WHO, Diphtheria Reference Unit) is as follows: A filter paper disc impregnated with diphtheria antitoxin (10 IU/mL) is placed on a horse serum agar plate. At least 10 colonies are spot-inoculated 7–9 mm away from the disc and incubated for 48 hours. The antitoxin diffuses out and forms precipitin bands between the disc and the bacterial

growth at the point where it meets the diphtheria toxin (diffusing out of the organisms).

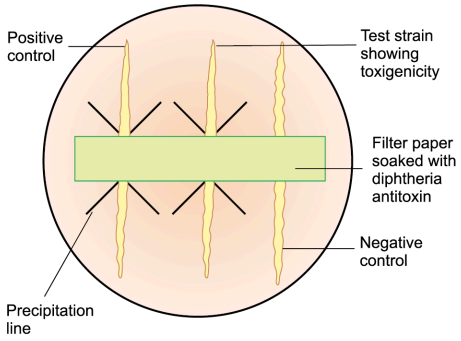


Fig. 23.4 Elek's gel precipitation test

- ***Tissue culture method*** The toxigenicity of *C. diphtheriae* can be demonstrated by incorporating its strains in the agar overlay of cell culture monolayers. The toxin produced by the organism diffuses into the cells below and causes lysis of the cells.
- ***PCR*** Polymerase chain reaction for the detection of the toxin gene (*tox*) in clinical isolates has been developed. This can also be used to detect the toxin from clinical material. This method is useful in patients already on antibiotics, in whom the organisms cannot be isolated.
- ***ELISA*** The test may be done to detect the toxin from the patient's isolate using antitoxin and enzyme–substrate system.
- ***Immunochromatography*** This method has also been employed to demonstrate the toxin within hours.

Epidemiology

Diphtheria has been virtually eradicated from most Western countries by effective prophylaxis and mass immunisation. In some developing countries where childhood immunisation programmes have not been implemented effectively, diphtheria continues to be a serious problem. In India, it is included in the universal immunisation programme for infants. However, sporadic cases are still reported. This could be due to non-adherence to childhood immunisation schedules.

Several adult diphtheria cases have also been reported in India. This could

be due to waning antibody levels or not having been immunised during childhood.

Cases of diphtheria in children and adults were reported as an aftermath of civil wars and social unrest. Incidences of outbreaks of adult diphtheria were reported in the 1990s when they caused a widespread epidemic in the Russian Federation and the former Soviet Republics.

The gravis and intermedius types are associated with high case fatality rates, while infections due to mitis are less lethal.

Paralytic complications are most common in gravis, hemorrhagic complications in gravis and intermedius, and **obstructive lesions** in the air passage in mitis infections.

In general, mitis is the predominant strain in endemic areas, while gravis and intermedius tend to be epidemic. The mitis type is able to establish a commensal relationship with the host unlike the more virulent types.

Evidence shows that the gravis and, to a lesser extent, the intermedius strains can spread more readily than mitis in populations naturally immune or artificially immunised.

Asymptomatic carriers who outnumber cases by a hundredfold or more in endemic areas are the most significant sources of infection. In the temperate regions, carriage is mainly in the nose and throat. Nasal carriers harbour the bacilli for longer periods than pharyngeal carriers.

Prophylaxis

Protective immunity

Immunisation is aimed at increasing protective levels of antitoxins (antibodies) in the circulation. The susceptibility of an individual is tested by determining the antitoxin level.

In vitro assays The circulating antitoxin level can also be determined by serological tests such as passive hemagglutination or by neutralisation in cell culture. **Antitoxin levels of more than 0.1 IU/mL of blood is considered an index of protective antibody level.**

Vaccines

Active immunisation Immunisation against diphtheria is done using the **toxoided** form of diphtheria toxin. Currently, two preparations are available

for active immunisation:

1. **Formol toxoid** (also known as fluid toxoid), which is prepared by incubating the toxin with formalin.
2. **Adsorbed toxoid,** which is prepared by purifying toxoid adsorbed onto insoluble aluminium phosphate, and less often, aluminium hydroxide. It is much more immunogenic than formol toxoid.

Dosage Diphtheria toxoid is usually given to children as a trivalent preparation containing tetanus toxoid and pertussis vaccine, as the **DTP, DPT or triple vaccine** as prescribed by the **Universal Immunisation Programme (UIP**). For young children, diphtheria toxoid is given in a dose of 10–25 Lf (limit of flocculation) units to all recommended individuals.

Schedule The schedule of primary immunisation of infants and children consists of three doses of DPT given at intervals of at least four weeks, preferably six weeks or more, followed by a fourth dose about a year afterwards. A further booster dose is given at school entry.

Adult immunisation Smaller doses (1–2 Lf units) are used for older children and adults to minimise adverse reactions**. In toxoid preparations, the lower dose of toxoid is indicated by the small letter 'd' and the full dose by capital 'D'. For example, the tetanus–diphtheria vaccine for adults containing low-dose diphtheria toxoid is referred to as 'Td'.**

Some **side effects** or adverse reactions which might occur after vaccination are injection site reactions (redness, warmth, swelling, tenderness, itching, pain, hives and rash), fever, drowsiness, fretfulness, vomiting, anorexia, persistent crying (in infants), and rarely, convulsions.

Passive immunisation This is an emergency measure to be used when susceptible persons are exposed to infection, as when a case of diphtheria is admitted to a general pediatric ward. It consists of the subcutaneous administration of 500–1,000 units of antitoxin (antidiphtheritic serum, ADS). As this is a horse serum, precaution against hypersensitivity should be observed.

Combined immunisation This consists of the administration of the first dose of adsorbed toxoid on one arm and ADS on the other arm, to be continued for the full course of active immunisation. Ideally, all persons who receive ADS prophylactically should receive combined immunisation.

Chemoprophylaxis
This is sometimes given to the susceptible close contacts of a diphtheria patient, with erythromycin or azithromycin along with the booster dose of the vaccine.

Treatment

Specific treatment of diphtheria consists of antitoxic and antibiotic therapy. **Antitoxin** should be given immediately when diphtheria is suspected, as the fatality rate increases with delay in starting antitoxic treatment. **The recommended dose is 20,000–100,000 units for serious cases; half the dose is given intravenously**. Antitoxin treatment is generally not indicated in cutaneous diphtheria as the causative strains are usually non-toxigenic.

C. diphtheriae is sensitive to **penicillin** and can be cleared from the throat within a few days of penicillin treatment. Diphtheria patients are given a course of penicillin as a supplement to and not instead of antitoxin therapy. In case of penicillin allergy, erythromycin or azithromycin may be given.

Erythromycin is more active than penicillin in the treatment of **carriers.**

OTHER PATHOGENIC CORYNEBACTERIA

Based on the improved growth on adding lipid to the medium, the other corynebacteria can be divided into:

- Non-lipophilic corynebacteria, e.g., *C. pseudotuberculosis* and *C. ulcerans*
- Lipophilic corynebacteria, e.g., *C. jeikeium* and *C. urealyticum*

Non-lipophilic Corynebacteria

Corynebacterium ulcerans is closely related to *C. diphtheriae* and can cause diphtheria-like lesions. It resembles the gravis type of the diphtheria bacillus. It produces two types of toxins, one probably identical to the diphtheria toxin and the other resembling the toxin of *C. pseudotuberculosis.* This organism has been found to cause infections in cows. Human infections may be transmitted through cow's milk. ***C. ulcerans*** is sensitive to erythromycin. Diphtheria antitoxin is protective.

Erythrasma, a localised infection of the stratum corneum usually affecting the axilla and groin, is **caused by *C. minutissimum*** which

produces superficial skin lesions. This is a lipophilic corynebacterium and can be grown readily in media containing 20% fetal calf serum.

Lipophilic Corynebacteria

Corynebacterium jeikeium is one of the most important members of this group and can cause cutaneous and bloodstream infections with a high mortality rate. This is often seen in immunocompromised hosts. This organism is usually multidrug-resistant, responding only to vancomycin. It is increasingly being associated with infective endocarditis.

Arcanobacterium haemolyticum (formerly *Corynebacterium haemolyticum*) can cause pharyngitis and skin ulcers.

DIPHTHEROIDS

Corynebacteria occur as normal commensals in the throat, skin, conjunctiva and other areas. These may sometimes be mistaken for diphtheria bacilli and are called diphtheroids. In general, **diphtheroids stain more uniformly than** *C. diphtheriae*, possess few or no metachromatic granules and tend to be arranged in parallel rows (palisades) rather than in a cuneiform pattern. However, some diphtheroids may be indistinguishable from *C. diphtheriae* microscopically. Differentiation is by biochemical reactions and more reliably, by virulence tests. The common diphtheroids are *C. pseudodiphtheriticum* (*C. hofmannii*), which is found in the throat and

C. xerosis, which is found in the conjunctiva. The former is urease-positive and does not ferment glucose, while the latter is urease-negative and ferments glucose. Both are pyrazinamidase-positive, unlike *C. diphtheriae.*

OTHER CORYNEFORM BACTERIA

Besides the genus *Corynebacterium*, a number of other genera of coryneform bacteria have been defined. Among them, the genus *Propionibacterium* is of medical interest. This is because three species, *P. acnes, P. granulosum* and *P. avidum*, are constantly present on human skin. They are anaerobic and aerotolerant, growing well in media containing lipid. *P. acnes* is often isolated from lesions of acne but its pathogenic role is uncertain.

Corynebacterium parvum, which is frequently used as an immunomodulator, is a mixture of the *Propionibacterium* species.

Important diseases and species of corynebacteria are summarised in Table 23.3.

Table 23.3 Important diseases and species of *Corynebacterium*

***Corynebacterium* species**	**Disease produced**
C. diphtheriae	Toxigenic strains—diphtheria Non-toxigenic strains—bacteremia, endocarditis
C. ulcerans	Toxigenic strains—diphtheria
C. pseudotuberculosis	Toxigenic strains—diphtheria
C. jeikeium	Infective endocarditis, bacteremia in immunocompromised hosts
C. urealyticum	Urinary tract infections
C. striatum	Health care-associated infections
Arcanobacterium hemolyticum	Pharyngitis, wound infections, septicemia

Key points

- *Corynebacteria* are non-motile, aerobic, gram-positive bacilli, some species being commensals in the human body.
- *Corynebacterium diphtheriae* causes diphtheria in the upper respiratory tract. Skin infections may occur due to toxigenic and non-toxigenic strains in individuals in poor socioeconomic circumstances.
- The bacterium attaches to the back of the throat of (mainly) children. This is followed by the secretion of the diphtheria toxin, which kills cells and causes inflammation and fibrin accumulation, leading to the formation of the characteristic pseudomembrane (which may break off and lead to the asphyxiation of the child).
- *Corynebacterium diphtheriae* grows slowly on selective media containing tellurite (colonies appear black) and rapidly on enriched media such as Loeffler's serum slope.
- Toxin production can be detected by Elek's gel precipitation test.
- Active immunisation during childhood with the diphtheria toxoid stimulates the production of neutralising antibodies that protect against the effects of active toxin secreted during infection.
- Treatment is by antitoxin and penicillin.

- Other pathogenic corynebacteria include *Corynebacterium jeikeium, C. pseudotuberculosis, C. ulcerans* and *C. minutissimum*.
- Diphtheroids resembling *C. diphtheriae* occur as normal commensals in the throat, skin, conjunctiva and other areas.

Questions

Essay

1. Describe the morphology, cultural characteristics and pathogenesis of *Corynebacterium diphtheriae* and add a note on prophylactic treatment.

Short Answers

1. Mechanism of action and detection of the diphtheria toxin
2. Diseases caused by the *Corynebacterium* species
3. Laboratory diagnosis of diphtheria

Part III: Bacteriology

Chapter 24

Bacillus

Chapter navigation

- *Bacillus anthracis*
- Anthrax
- *Bacillus cereus*
- Food poisoning

INTRODUCTION

The genus *Bacillus* includes gram-positive bacilli that produce spores. Members of this group exhibit great diversity in their properties. The genus includes **psychrophilic, mesophilic and thermophilic** species, with maximum temperatures for vegetative growth ranging from about 25°C to above 75°C and the minimum from about 5°C to 45°C. Their salt tolerance varies from less than 2% to 25% NaCl. Their heat-resistant spores, some which are killed only at 120°C, may be found in soil, dust, water and air. They constitute the commonest contaminants in bacteriological culture media.

- *Bacillus anthracis*, the causative agent of anthrax (*Clinical case*) is a major pathogen. Anthrax is primarily a disease of cattle and sheep and may affect other animals also. Humans get infected when they come into contact with spores from any of these sources.
- *B. cereus* can cause food-borne gastroenteritis. Some species may also be responsible for opportunistic infections.

B. anthracis was the first pathogenic bacterium to be observed under the microscope (Pollender, 1849), the first communicable disease shown to be transmitted by inoculation of infected blood (Davaine, 1850), the first bacillus to be isolated in pure culture and shown to possess spores (Koch, 1876) and the first bacterium used for the preparation of an attenuated vaccine (Pasteur, 1881).

Clinical Case

A 45-year-old cowherd presented with a black eschar on his left hand. There was extensive swelling around the ulcer but it was not painful. There were a few vesicular lesions surrounding the ulcer. He gave a history of handling a dead animal on his farm. The fluid from the vesicle was positive for gram-positive bacilli. Culture was positive for Bacillus anthracis, establishing a diagnosis of cutaneous anthrax. The patient responded to seven days' treatment with ciprofloxacin.

BACILLUS ANTHRACIS MORPHOLOGY

B. anthracis is an aerobic, gram-positive, non-motile, sporulating bacillus which measures 1–1.5 × 3–5 ∝m.

In clinical material and culture, it appears in chains (end to end) and has the appearance of a bamboo stick or boxcar (Fig. 24.1). It grows readily on conventional microbiology media, including blood agar. Spores are not generally seen on direct Gram stain of specimens but they appear in culture when nutrients are depleted. The usual time for growth is 6–24 h. The organism is easily recovered if samples are obtained before the administration of antibiotics.

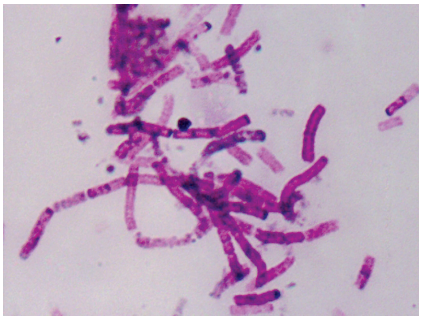


Fig. 24.1 Bamboo stick appearance in Gram stain (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Spores

Sporulation occurs under unfavourable conditions. Oxygen is required for the formation of spores but not for their germination. Spores are central or subterminal and either elliptical or oval but do not bulge beyond the diameter of the vegetative cell.

Spores are highly resistant to physical and chemical agents. They have been isolated from naturally infected soil after as long as 60 years.

- They resist dry heat at 140°C for 1–3 hours and boiling for 10 minutes.
- **Spores are formed in culture or in the soil but never in the animal body during life**.
- **Vegetative bacilli**, on the other hand, are found in tissues and are destroyed at 60°C in 30 minutes.

The optimum temperature for sporulation is 25°C to 30°C.

Disinfection of wool is done by '**duckering**', in which formaldehyde is used as a 2% solution at 30–40°C for 20 minutes and as 0.25% solution at 60°C for six hours for the disinfection of animal hair and bristles. In the carcasses of animals that have died of anthrax, the bacilli remain viable in the bone marrow for a week and in the skin for two weeks. **Normal heat fixation of smears may not kill the bacilli in blood films.**

Pathogenicity

Two **virulence factors** have been identified in the pathogenesis:

Capsule The **capsule** is a polypeptide composed of a polymer of d(–) glutamic acid. It is plasmid-borne. It aids virulence by inhibiting phagocytosis. **Loss of the plasmid leads to loss of virulence. This attenuation is used in the making of anthrax spore vaccine (Sterne vaccine)**.

Anthrax toxin The anthrax toxin is encoded by a separate plasmid. The original anthrax vaccine developed by Pasteur may have been made up of bacilli that had lost this plasmid.

The toxin is a complex of **three fractions:**

- **Protective antigen factor (PA or Factor II)** PA is the binding fraction on the target cell surface, which provides attachment sites for Factor I or Factor III, facilitating their entry into the cell. The antibody to PA is protective.
- **Edema factor (OF or Factor I)** OF is an adenyl cyclase activated only inside the target cells, leading to intracellular accumulation

of cyclic AMP. This is believed to be **responsible for edema and the other biological effects of the toxin.**

- **Lethal factor (LF or Factor III)** Entry of LF into the target cell causes cell death but the mechanism of this action is not known.

Anthrax

Anthrax is a zoonotic infection. Animals are infected by ingestion of the spores present in the soil. The disease is a fatal septicemia in animals but may sometimes be localised, resembling the cutaneous disease in humans. Infected animals shed large numbers of bacilli from the mouth, nose and rectum. These sporulate in the soil and remain as the source of infection. The spores remain viable for many years.

Human anthrax is contracted from animals. The disease may be: a) cutaneous, b) pulmonary or c) intestinal, all types leading to fatal septicemia or encephalopathy and meningitis.

Clinical Features

1. Cutaneous anthrax

Common sites of entry of the infection are the face, neck, hands, arms and back (exposed parts of the body). The lesion is called a **malignant pustule**.

The lesion starts as a papule 1–3 days after infection and becomes vesicular, containing fluid which may be clear or bloodstained with several satellite lesions (*Clinical case*).

The whole area is congested and edematous (Fig. 24.2), and is covered by a black **eschar.** (The name *anthrax*, which means coal, comes from the black colour of the eschar.)

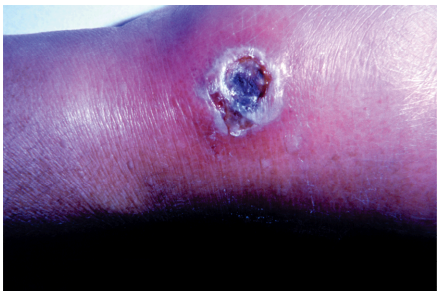


Fig. 24.2 Cutaneous lesion (malignant pustule) with black central eschar and edematous raised margin; satellite lesions are seen (*Source:* Public Health Image Library, ID 20334 / F. Marc LaForce, M.D. / CDC)

Hide porter's disease The disease used to be common in dock workers carrying loads of hides and skins on their bare backs. Cutaneous anthrax

generally resolves spontaneously, but 10–20 per cent of untreated patients may develop fatal septicemia or meningitis.

2. Pulmonary anthrax

This is called the **wool-sorter's disease** because it used to be common among those who worked in wool factories where they would inhale spores from infected wool. Hemorrhagic pneumonia and dissemination into the meninges can also occur.

3. Intestinal anthrax

This occurs mainly in communities that consume undercooked or uncooked meat.

Severe enteritis with bloody diarrhea occurs, ending fatally if left untreated.

4. Anthrax meningitis or meningoencephalitis

This is a fatal infection which occurs in the CNS via the bloodstream. **The CSF is characteristically hemorrhagic and the condition may be mistaken for cerebrovascular accident**.

Human anthrax may be:

- **Industrial**—found in workers in industries such as meat packing or wool industries.
- A**gricultural**—seen among animal handlers, meat packers and wool sorters.
- **Non-industrial**—often an occupational disease among those who associate frequently with animals, such as veterinarians, butchers and farmers.

***Stomoxys calcitrans* and other biting insects may occasionally transmit infection mechanically.**

Epidemiology

Anthrax is enzootic in India; there have been frequent reports of anthrax around the Andhra Pradesh–Tamil Nadu border. These infections are cutaneous and meningoencephalitic human infections, with high mortality rate. There have been outbreaks in Karnataka and West Bengal. Anthrax

infection in human beings provides permanent immunity and second attacks are extremely rare.

Bioterrorism
B. anthracis is a potential tool in biological warfare and was used for this purpose in 2001, when it was sent by mail to various destinations in the USA, causing disease and many deaths.

Laboratory Diagnosis

Biosafety
Anthrax is a highly virulent organism. It requires processing in biosafety level 3 laboratories equipped to protect against aerosols. When an animal is suspected to have died of anthrax, autopsy is not permissible, as the spilt blood will lead to contamination of the soil. **Biosafety level Class II/III cabinets** are mandatory to handle such specimens.

A suspected case of anthrax may be provisionally diagnosed in the laboratory by the following methods:

1. **Specimen**

- **Clinical specimens** Common specimens are pus from the cutaneous lesions, blood and CSF in case of bloodstream invasion and meningoencephalitis, sputum in cases of pulmonary anthrax and feces in the case of gastroenteritis. All clinical samples must be treated as potentially biohazardous.
- **In animals** A ear may be cut off from the carcass and sent to the laboratory. Alternatively, swabs soaked in blood or several blood smears may be sent.

Confirmation of the isolate needs to be done in laboratories equipped with Class II/III biosafety cabinets.

2. **Microscopy** The demonstration of gram-positive bacilli with the morphology of anthrax bacilli (Fig. 24.3) in clinical material, and the presence of spores in culture isolates can help in diagnosis. The spores do not stain by ordinary methods but can be stained differentially by special techniques:

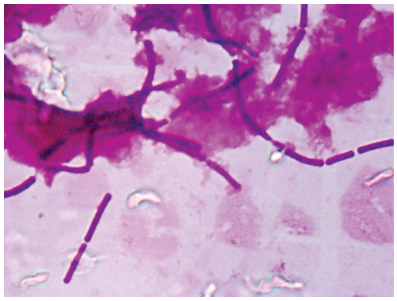


Fig. 24.3 *Bacillus anthracis* in blood: gram-positive rods, showing bamboo stick appearance in smear from automated blood culture bottle (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- **M'Fadyean reaction with polychrome methylene blue** When a blood film containing anthrax bacilli is stained with **polychrome methylene blue** for a few seconds and examined under the microscope, an amorphous purplish material is noticed around the bacilli. This represents the capsular material. This is the **M'Fadyean reaction** and is employed in the presumptive diagnosis of anthrax in animals.
- **Sudan black B stain** Fat globules may be made out within the bacilli.
- **Immunofluorescent microscopy** Specific immunoglobulin tagged with fluorescent stain can be used to confirm the identification.

3. **Culture** Isolation of the bacillus is easy as they grow on most laboratory media with a temperature range of 12–45°C (optimum 35–37°C).

- On **agar plates,** irregularly round colonies are formed, 2–3 mm in diameter; they are raised, dull, opaque and greyish-white with a frosted glass appearance. On blood agar, the colonies are non-hemolytic, though occasional strains produce a narrow zone of hemolysis. Under a low-power microscope, the edge of the colony is seen to be composed of long, interlacing chains of bacilli, resembling locks of matted hair. This is called the **Medusa head appearance** (Fig. 24.4). **This should not be attempted in the laboratory as the cultures are highly infectious due to the presence of spores.**

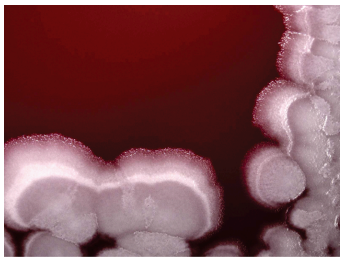


Fig. 24.4 Non-hemolytic dry colonies of *B. anthracis* described as Medusa head appearance on blood agar (*Source:* Public Health Image Library, ID 17099 / Todd Parker, Ph.D., Assoc Director for Laboratory Science, Div of Preparedness and Emerging Infections at CDC / CDC)

- When *B. anthracis* is grown on a **solid medium** containing 0.05–0.50 units of penicillin/mL, the cells become large and spherical within 3–6 hours and arrange themselves in chains on the surface of the agar, resembling a string of pearls. This '**string of pearls reaction**' clearly differentiates *B. anthracis* from *B. cereus* and other aerobic spore bearers.
- Virulent capsulated strains form rough cultures, while avirulent or attenuated strains form smooth colonies.
- On **gelatin stab culture,** a characteristic '**inverted fir tree**' appearance is seen. In broth, growth occurs as floccular deposits, with little or no turbidity.

Another useful test of differentiation is the susceptibility of *B. anthracis* to gamma phage.

- A **selective medium (PLET medium**) consisting of polymyxin, lysozyme, ethylene diamine tetraacetic acid (EDTA) and thallous acetate added to heart infusion agar has been devised to isolate *B. anthracis* from mixtures containing other spore-bearing bacilli.

4. **Animal inoculation** Experimentally, most animals are susceptible, including guinea pigs, rabbits and mice.

5. **Serological demonstration** Diagnosis may be established by Ascoli's thermoprecipitin test which demonstrates the anthrax antigen in tissue extracts. **Direct fluorescent antibody test (DFA)** for capsule-specific staining and for polysaccharide cell wall antigen are now available.

6. **Serology for antibodies** Acute and convalescent phase sera should be obtained, since antibodies to the organism can be demonstrated by gel

diffusion, complement fixation, antigen-coated tanned red cell agglutination and ELISA techniques.

7. **Molecular typing** For epidemiological studies and strain characterisation, **MLVA** (**multiple-locus variable number tandem repeat analysis**) or **AFLP** (**amplified fragment length polymorphism**) can be used. These methods help in tracking the source in case of an outbreak or to monitor the spread of infection across the globe.

CDC guidelines for identification after bioterrorism threat

After the bioterrorism incidents in the USA in 2001, the **Centres for Disease Control and Prevention (CDC)** prepared **guidelines** for the identification of the anthrax bacillus.

> Any large gram-positive bacillus with the general morphology and cultural features of anthrax—non-motile, non-hemolytic on blood agar and catalase positive—can be given a presumptive report of anthrax.

For initial confirmation, lysis by gamma phage and direct fluorescent antibody test (DFA) for capsule-specific staining and for polysaccharide cell wall antigen are sufficient.

Biosafety and Prevention

1. **General methods** Prevention of human anthrax is mainly by general methods such as improvement of factory hygiene and proper sterilisation of animal products like hides and wool. Carcasses of **animals suspected to have died of anthrax should be buried deep in quicklime** or cremated to prevent soil contamination. The processing and isolation should be done in designated reference laboratories, generally in the veterinary facilities.

2. **Immunisation** **Anthrax Vaccine Adsorbed (AVA) is the licensed vaccine for human use**. AVA BioThrax is a vaccine for human use obtained from the culture of
B. anthracis having the protective antigen. Personnel at risk including veterinarians and animal handlers are generally vaccinated to prevent occupational exposure.

- **Pre-exposure** Only high-risk individuals are given five doses of the vaccine, followed by boosters.

- **Post-exposure** It is given after exposure to inhalation anthrax along with antibiotics.

Vaccines are available and used more commonly for prevention of anthrax in animals by active immunisation.

i) ***Pasteur's anthrax vaccine*** It is of great historical importance. It was Pasteur's convincing demonstration of the protective effect of his anthrax vaccine in a public experiment at Pouilly-le-Fort in 1881 that marked the beginning of scientific immunoprophylaxis.

ii) ***Sterne vaccine*** It consists of spores of attenuated strains of a non-capsulated, avirulent, mutant strain. It is used for animals.

ii) ***Mazzucchi vaccine*** It contains spores of stable, attenuated Carbazoo strain in 2% saponin.

Treatment

CDC has issued updated guidelines on anthrax post-exposure prophylaxis (PEP) and treatment. Its recommendations include the following:

1. Uncomplicated cutaneous anthrax can be treated with a single oral agent—fluoroquinolone or doxycycline for 7–14 days.
2. For patients suspected to have systemic anthrax, antitoxin should be added to combination antimicrobial drug treatment.
3. Antitoxin (antibody against protective antigen) is used with antibiotics. These include:

    - Human anthrax immunoglobulin (Anthrasil)
    - Monoclonal antibodies to protective antigen—Obiltoxaximab or Roxibacumab

These are indicated in severe cases with systemic involvement such as inhalational anthrax because antibiotics have no effect on the toxin once it is formed.

4. Anthrax meningitis should be treated with at least three antimicrobial drugs including clindamycin and a full 60 days of antibiotic treatment, regardless of the vaccine strains.

5. **Post-exposure prophylaxis (PEP)** for inhalation anthrax in adults—ciprofloxacin and doxycycline are first-line treatments for 60 days along with vaccine (AVA BioThrax).

ANTHRACOID BACILLI

A large number and variety of non-pathogenic, aerobic, spore-bearing bacilli that appear as common contaminants in cultures and have a general resemblance to the anthrax bacilli have been collectively called pseudoanthrax or anthracoid bacilli. Table 24.1 lists the main differentiating features of anthrax and anthracoid bacilli.

Table 24.1 Differentiating features of anthrax and anthracoid bacilli

Anthrax bacilli	Anthracoid bacilli
Non-motile	Generally motile
Capsulated	Non-capsulated Grow in short chains
Produce Medusa head colonies	Do not produce Medusa head colonies
No growth in penicillin agar (10 units/mL)	Usually grow in penicillin agar
Hemolysis is absent or weak	Hemolysis is usually well-marked
Inverted fir tree growth and slow gelatin liquefaction	Rapid gelatin liquefaction
No turbidity is noted in broth	Usually produce turbidity in liquid culture
Salicin fermentation negative	Salicin fermentation usually positive
No growth at 45°C	Usually grows at 45°C
Growth inhibited by chloral hydrate	Growth not inhibited by chloral hydrate
Susceptible to gamma phage	Not susceptible to gamma phage
Pathogenic to laboratory animals	Not pathogenic to laboratory animals

BACILLUS CEREUS

B. cereus is an important cause of food poisoning. It is widely distributed in nature and may be readily isolated from soil, vegetables and a wide variety of foods including milk, cereals, spices, meat and poultry. *B. cereus* is generally motile but non-motile strains may occur. It is not capsulated and

not susceptible to gamma phage.

Patterns of Food-Borne Disease

Two types of food poisoning have been associated with *B. cereus:*

1. Diarrheic type
2. Emetic type

The **diarrheal type** is associated with a **wide range of foods** including cooked meat and vegetables. It is characterised by diarrhea and abdominal pain, 8–16 hours after ingestion of contaminated foods. Vomiting is rare. *B. cereus* is not found in large numbers in the fecal specimens from these patients. **The diarrheal disease is mostly caused by serotypes 2, 6, 8, 9, 10 or 12.**

The **emetic type** is associated almost exclusively with the **consumption of cooked rice**, usually fried rice from Chinese restaurants. The illness is characterised by acute nausea and vomiting, 1–5 hours after the meal. Diarrhea is not common. *B. cereus* is present in large numbers in the cooked rice and fecal samples from these patients. This is caused by **serotypes 1, 3 or 5.** The emetic toxin is produced only when *B. cereus* is grown in rice but not in other media.

Two mechanisms of action have been described for the action of the enterotoxin of *B. cereus*, one involving the stimulation of the cyclic adenosine monophosphate (cAMP) system and the other independent of it.

Diagnosis

Special selective media — (i) mannitol egg yolk phenol red polymyxin agar (**MYPA**) and (ii) polymixin-pyruvate mannitol bromothymol blue agar **(PEMBA)** — are selective media useful in isolating *B. cereus* from feces and food sources. *B. cereus* produces lecithinase and ferments glucose but not mannitol. **The presence of 10^5 or more bacteria per gram of specimen is diagnostic.**

Treatment

Both types of illness are mild and self-limiting, requiring no specific treatment.

BACILLUS THURINGIENSIS

Bacillus thuringiensis is found in the soil. It has been exploited as a biological pesticide and larvicidal agency. It produces a toxin that is fatal to mosquitoes and other herbivorous insect larvae, hence used in their control. *B. thuringiensis* is also the source of genes used to genetically modify food crops to make them resistant to pests.

BACILLUS SPECIES AS STERILISATION INDICATORS (CONTROLS)

Many *Bacillus* species are used as biological indicators in sterilisation controls, e.g., spores of *B. atrophaeus (subtilis* var *niger)* and *Geobacillus stearothermophilus* (*see* Chapter 3).

Key points

- Bacteria of the genus *Bacillus* are ubiquitous organisms found in the soil, water, airborne dust and even human intestines. These aerobic, gram-positive bacilli form spores and are catalase-positive. *B. anthracis* is non-motile, but other species, including *Bacillus cereus*, are motile.
- *Bacillus anthracis* causes anthrax, a zoonotic infection. Anthrax manifests as cutaneous type (black lesion, malignant pustule or black eschar), pulmonary type or gastrointestinal type.
- Diagnosis is by microscopy: staining with characteristic M'Fadyean reaction is employed for the presumptive diagnosis of anthrax in animals.
- In culture on blood agar plates, large, spreading, grey-white, non-hemolytic colonies with irregular margins are formed (Medusa head colonies). The organisms are non-motile and appear to have square ends attached by joints to other cells (bamboo stick appearance).
- Exposure to spores in hides of domestic livestock (goats) leads to zoonotic infection. Deep burial in lime pits or burning of animal carcasses is recommended.
- Exposed persons must be treated with ciprofloxacin or doxycycline; vaccination against PA is recommended where exposure is a risk. Antitoxin is indicated in severe disease.
- *Bacillus cereus* occurs worldwide. It causes food poisoning, manifested by vomiting and diarrhea.
- The enterotoxin may be pre-formed in stool, causing emetic type of presentation, or produced in the intestines, causing diarrheal type of illness.

Questions

Essays

1. Describe the pathogenesis and laboratory diagnosis of anthrax.
2. Describe the pathogenesis of food poisoning due to *B. cereus*.

Short Answers

1. M'Fadyean reaction
2. Virulence factors of *B. anthracis*
3. Malignant pustule

Part III: Bacteriology

Chapter 25

Actinomycetes

Chapter navigation

- *Actinomyces*
- *Nocardia*
- Actinomycotic mycetoma

INTRODUCTION

Actinomycetes are slender, gram-positive bacteria that possess a cell wall containing muramic acid. They bear a superficial resemblance to fungi as they form branching filaments. Actinomycetes are related to mycobacteria and corynebacteria. They are non-motile, non-sporing, non-capsulated filaments that break up into bacillary and coccoid forms. Most are free-living, particularly in the soil.

Actinomycetes may be classified as:

- **Anaerobic/Microaerophilic** *Actinomyces*, *Bifidobacterium*, *Arachnia* and *Rothia*
- **Aerobic** *Nocardia, Actinomadura*, *Dermatophilus* and *Streptomyces*

ACTINOMYCES

Actinomycosis

Bollinger (1877) found a mould-like organism in the lesion of 'lumpy jaw' (actinomycosis) in cattle. The name *Actinomyces bovis* was coined by Harz in 1878 (*Actinomyces* in Greek means 'ray fungus'). Wolf and Israel (1891) isolated an anaerobic bacillus from human lesions. This was named *Actinomyces israelii*, while actinomyces causing infection in cattle was

named as *A. bovis*.

The disease is a chronic granulomatous infection occurring in human beings and animals. It is characterised by the development of indurated swellings, mainly in the connective tissue, suppuration and the discharge of pale granules called **sulphur granules**. The lesion often has multiple sinuses.

Actinomycosis in human beings is an endogenous infection. The *Actinomyces* is normally present in the mouth, intestine and vagina as a commensal. Trauma, foreign bodies or poor oral hygiene may favour tissue invasion. *A. israelii* is the most common causative agent. However, other actinomycetes such as *A. naeslundii, A. viscosus, A. odontolyticus, A. meyeri, A. gerencsonei* and *Propionibacterium propionicum* may sometimes be responsible. Actinomycosis is usually a mixed infection which may include *Bifidobacterium dentium, Actinobacillus actinomycetemcomitans, Eikenella corrodens, Haemophilus aphrophilus, Bacteroides*, fusobacteria, staphylococci and anaerobic streptococci.

Clinical Forms

There are four main clinical forms:

1. **Cervicofacial,** with indurated lesions on the cheek and submaxillary regions (*Clinical case* 1).
2. **Thoracic,** with lesions in the lung that may involve the pleura and pericardium and spread outwards through the chest wall.
3. **Abdominal,** where the lesion is usually around the cecum, involving the neighbouring tissues and the abdominal wall. Sometimes, the infection spreads to the liver via the portal vein.
4. **Pelvic,** where infections are associated with the intrauterine devices. Generally, abscesses are produced in the bone and soft tissue, with chronic draining sinuses to the exterior.

Actinomyces have been incriminated in inflammatory diseases of the gums (gingivitis and periodontitis) and sublingual plaques leading to caries. **Actinomycosis may also cause mycetoma** (see below).

Clinical Case 1

A 20-year-old woman presented to the surgery outpatient department with a soft, painless swelling on her left jaw, which was covered with exudate from a discharging sinus. She gave a history of trauma to

the jaw. On macroscopic examination of the pus, a light coloured granule was found in the exudate. Gram stain of the granule showed the presence of filamentous, branching, gram-positive bacilli. Cervicofacial actinomycosis was diagnosed. She was treated with intravenous penicillin G (8 million units per day) for 2 weeks followed by oral penicillin.

Sulphur granules are found in the exudate. The morphology in diseased tissue is of a long, branching mycelium with attached 'sulphur granules'.

Treatment includes surgical drainage or excision, together with large doses of penicillin for several weeks or months.

Laboratory Diagnosis

1. **Clinical specimen** Pus, granules, tissue or sputum are collected as specimens in pulmonary disease.

2. **Gross examination of granules** The granules are white or yellowish and range in size from minute specks to about 5 mm. Granules may be obtained by applying gauze pads over the discharging sinuses.

3. **Microscopy** Granules are crushed between two slides, stained with Gram stain and examined. A dense network of thin, gram-positive filaments, surrounded by a peripheral zone of swollen, radiating, club-shaped structures, presenting a sun's ray appearance are seen in the smear and tissue section (Fig. 25.1a and b).

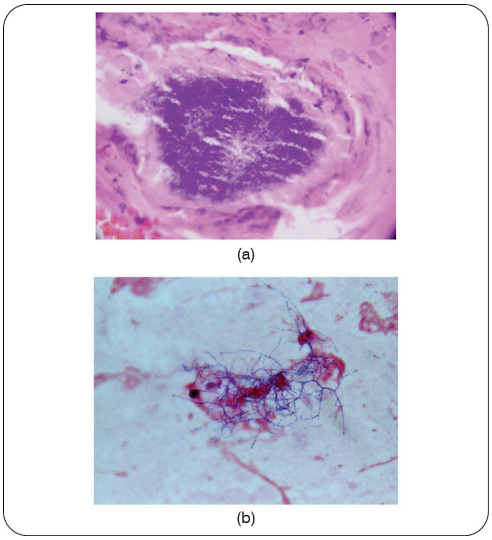


Fig. 25.1 (a) Sulphur granules in section with H and E stain and (b) gram-positive filamentous bacilli in pus

4. **Isolation in culture** Sulphur granules or pus-containing actinomycetes are washed and inoculated into thioglycolate liquid medium or streaked on brain–heart infusion agar and incubated anaerobically at 37°C. In thioglycolate, *A. bovis* produces general turbidity, whereas ***A. israelii* grows as fluffy balls at the bottom of the tube**. On solid media, *A. israelii* produces small, 'spidery colonies' in 48–72 hours that become heaped up, white and irregular or smooth, large colonies in 10 days. Other species have different types of colonies.

5. **Identification of species** The isolate is identified by microscopy, biochemical reactions and fluorescent antibody methods. Gel diffusion and immunofluorescence can differentiate *A. israelii* from other actinomycetes species and from other filamentous anaerobes that may produce granules in tissues.

Epidemiology

Actinomycosis occurs throughout the world but its incidence in developed countries has been declining, probably as a result of the widespread use of antibiotics. It is more common in rural areas and among agricultural workers. About 60 per cent of the cases are cervicofacial and 20 per cent are abdominal. Pelvic actinomycosis is seen mainly in women using intrauterine devices.

Treatment

The disease responds to prolonged treatment with penicillin or tetracycline. Treatment is continued for several months and supplemented by surgery where necessary.

NOCARDIA

Clinical Case 2

A 60-year-old, HIV-positive man developed pneumonia with high-grade fever and productive cough. His CD4 count was <200/mm^3. A diagnosis of pulmonary tuberculosis was ruled out by the repeatedly negative sputum smear. Further examination of sputum showed the presence of fine, branching filaments which were acid-fast (to 1% sulphuric acid). Culture on blood agar showed the growth of dry, buff-coloured colonies identified as N. asteroides. The patient responded to treatment with cotrimoxazole and amikacin.

Nocardia resembles actinomycetes morphologically but is aerobic. All species are gram-positive, and some such as *N. asteroides* and *N. brasiliensis* are also acid-fast to 1% sulphuric acid (Kinyoun method). They are frequently found in the soil. They produce cutaneous, subcutaneous or systemic lesions in humans. Pathogenic species are *N. asteroides, N. brasiliensis* and *N. caviae*.

Morphology

Nocardia are filamentous, rod-shaped bacteria that are non-motile and non-sporulating, gram-positive and weakly acid-fast (by **Kinyoun's acid-fast staining technique).** The organisms are obligate aerobes, and growth can often be enhanced by carbon dioxide. *N. asteroides* is the most commonly involved species in human disease. It is found worldwide in the soil and can infect animals. Transmission is thought to be through contaminated soil, not from animals or infected humans.

Clinical Forms

Clinical manifestation of *Nocardia* may be as follows:

1. **Cutaneous infection** may lead to local abscesses, cellulitis or lymphocutaneous lesions. The subcutaneous lesion is actinomycotic mycetoma (described below).
2. **Systemic nocardiosis,** which is usually caused by *N. asteroides,* manifests primarily as pulmonary disease, pneumonia, lung abscess or other lesions resembling tuberculosis (*Clinical case 2*).
3. **Metastatic manifestations** may involve the brain, kidneys and other organs. Systemic nocardiosis occurs more often in immunodeficient persons.

Laboratory Diagnosis

1. **Direct microscopy** Demonstration of partially acid-fast, branching, beaded filaments in smears from patient tissue is diagnostic of *Nocardia* infection.

2. **Isolation in culture** *Nocardia* are aerobic and grow readily on ordinary media, forming dry, granular, wrinkled colonies, which produce pigments ranging from yellow to red. The species can be identified by biochemical

tests.

Treatment

These organisms are resistant to penicillin. The most successful agents of treatment have been sulphonamides, particularly sulphamethoxazole and trimethoprim (cotrimoxazole). Surgery may be required to remove mycetomas. Therapy is most successful with skin and lung infections. Cotrimoxazole or minocycline given for several months may be useful. In immunocompromised patients, amikacin and cefotaxime are advisable in addition.

MYCETOMA (BACTERIAL)

Bacterial mycetomas are usually caused by actinomycetes—*Actinomyces (A. israelii, A. bovis), Nocardia (N. asteroides, N. brasiliensis, N. caviae), Actinomadura (A. madurae, A. pelletierii)* and *Streptomyces (S. somaliensis).*

Staphylococcus aureus and other pyogenic bacteria may occasionally cause a mycetoma-like lesion (botryomycosis).

Actinomycotic Mycetoma

Mycetoma is a localised, chronic, granulomatous involvement of the subcutaneous and deeper tissues, commonly affecting the foot and less often, the hand and other parts, and presenting as a tumour with multiple discharging sinuses (Fig. 25.2). This clinical syndrome was first described from Madura by Gill (1842) and came to be known as **Maduramycosis**. Mycetomas are usually caused by fungi but may be caused by bacteria as well.

Fig. 25.2 Actinomycotic mycetoma of the upper limb—multiple discharging sinuses with exudation of pus and granules (*Source:* Public Health Image Library, ID 14741 / Dr. Libero Ajello / CDC)

The etiological diagnosis of mycetoma is important in choosing the appropriate treatment. The colour of the granules gives some indication. In actinomycotic mycetoma, the granules are white to yellow, while in eumycotic mycetomas, the granules are generally black. Examination of smears of the crushed granules helps to differentiate actinomycotic from mycotic mycetomas. In the former, the filaments are thin (about 1 ∝m), while in the latter, they are stout (about 4–5 ∝m). Isolation of the agent in culture establishes the diagnosis.

Actinomycetes and hypersensitivity pneumonitis
Spores of some thermophilic actinomycetes such as the *Faenia* and *Saccharomonosporia* species present in mouldy hay, which when inhaled, may induce allergic alveolitis leading to chronic obstructive pulmonary disease (COPD, farmer's lung).

Key points

- Actinomycetes are gram-positive, non-motile, non-sporing, non-capsulated bacteria that form a mycelial network of branching filaments. They are thin, possess a cell wall containing muramic acid, have prokaryotic nuclei and are susceptible to antibacterial antibiotics.
- *Actinomyces*, is anaerobic or microaerophilic and non-acid-fast. It produces cervicofacial, pulmonary or abdominal actinomycotic infections.
- *Nocardia* species are aerobic and may be acid-fast (to 1% sulphuric acid). They produce pulmonary infections in immunocompromised patients
- Actinomycotic mycetoma is caused by *Actinomyces*, *Actinomadura*, *Nocardia* and *Streptomyces*.

Questions

Essay

1. Describe the etiology and laboratory diagnosis of actinomycosis.

Short Answers

1. Mycetoma
2. Sulphur granules

Part III: Bacteriology

Chapter 26

Clostridia

Chapter navigation

- *Clostridium perfringens*
- *Clostridium septicum*
- *Clostridium novyi (C. oedematiens)*
- *Clostridium histolyticum*
- *Clostridium tetani*
- *Clostridium botulinum*
- *Clostridium difficile*

INTRODUCTION

Anaerobic organisms are ubiquitous in nature. They constitute a major part of the normal flora of the human body, colonising the skin and mucous membrane. Anaerobic bacteria outnumber aerobic bacteria in many habitats, including most sites of the human body, especially the gastrointestinal tract. Even in such seemingly aerobic locations as the mouth and skin, anaerobic bacteria are ten to thirty times more common than aerobes. In the human intestines, they outnumber aerobic bacteria by a thousand-fold. The number of anaerobes present has been estimated to be 10^4–10^5/mL in the small intestine, 10^8/mL in saliva and 10^{11}/g in the colon.

Some anaerobes (e.g., Clostridia) can sporulate and their spores can be found in soil and excreta. They produce infections when they gain entry into sterile body sites.

Anaerobic bacteria of human importance are classified as shown in Table 26.1.

Table 26.1 Anaerobic bacteria of medical interest

Anaerobic spore-forming rods	Anaerobic non-spore-forming rods
Gram-positive rods • *Clostridium tetani* • *Clostridium perfringens*	**Gram-positive rods** • *Actinomyces* • *Eubacterium*

• *Clostridium botulinum*	• *Bifidobacterium* • *Lactobacillus* • *Propionibacterium*
	Gram-negative rods • *Bacteroides* • *Fusobacterium*
	Gram-positive cocci • *Peptostreptococcus* • *Peptococcus*
	Gram-negative cocci • *Veillonella* • *Acidaminococcus*

CLOSTRIDIA

Clostridia are gram-positive anaerobic bacteria which form heat-resistant spores. Some of the species are found in the gut of humans and animals as commensal flora. Pathogenic organisms can cause **gas gangrene, tetanus, botulism, food poisoning** and **pseudomembranous colitis**. They act mainly by producing **potent exotoxins.**

Classification

Molecular Based on 16S rRNA gene sequences, 19 clusters have been identified so far, of which, **clinically relevant species belong to cluster 1.**

Phenotypic Classification based on morphology, culture and biochemicals is commonly used in clinical laboratories.

Morphology

Clostridia are highly pleomorphic, usually 3–8 × 0.4– 1.2 ∝m in size, gram-positive rods. Clostridia are motile with peritrichate flagella, the exceptions being ***C. perfringens*** and ***C. tetani*** **type VI which are non-motile**. Motility is described as 'stately' or slow. *C. perfringens* and *C. butyricum* are capsulated, while others are not.

Spores

Clostridial spores are wider than the bacillary bodies, giving the bacillus a swollen appearance resembling a spindle—hence, the name *Clostridium* (from *kloster*, meaning 'spindle') (Fig. 26.1). Some (such as *C. sporogenes*)

sporulate readily while others (such as
C. perfringens) do so inconsistently. The shape and position of spores vary in different species and are used in the identification and classification of clostridia (Fig. 26.2). The spore may be:

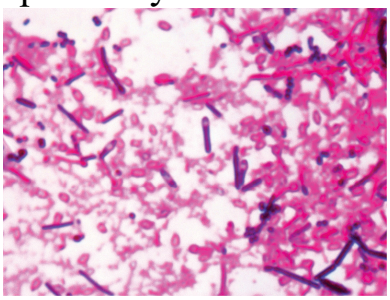


Fig. 26.1 Clostridial spores wider than the bacillary body; spindle shape and drumstick appearance (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

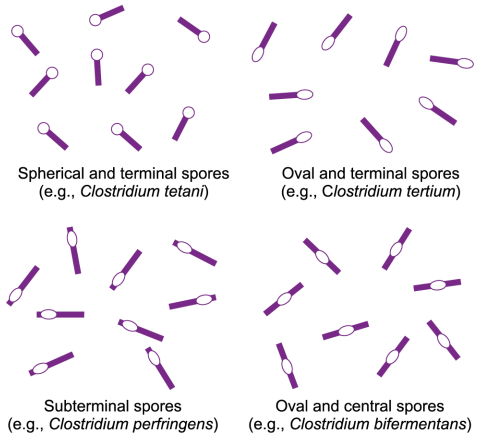


Fig. 26.2 Line diagrams depicting the types of clostridial spores

- **Central or equatorial**, giving the bacillus a spindle shape (*C. bifermentans*)
- **Subterminal**, the bacillus appearing club-shaped (*C. perfringens*)
- **Oval and terminal**, resembling a tennis racket (*C. tertium*)
- **Spherical and terminal**, giving a drumstick appearance (*C. tetani*)

Resistance in spores

Table 26.2 summarises the viability of the spores.

Table 26.2 Viability of spores of clostridia

C. botulinum spores	Survive 100°C up to 3–4 hours At 105°C, they are not killed

	completely in less than 100 minutes
C. perfringens spores Spores of Type A strains (cause food poisoning)	Destroyed by boiling for less than five minutes Survive for several hours
C. tetani spores	Persist for years in dried earth Resist boiling for 15–90 minutes
Spores of all species are killed by autoclaving at 121°C within 20 minutes	
• Spores are resistant to phenolic disinfectants • Halogens are effective in killing spores • 1% aqueous iodine solution kills spores within three hours • Glutaraldehyde (2% at pH 7.5–8.5) is very effective in killing the spores	

Clostridia of medical importance may also be categorised based on the diseases they produce (Table 26.3).

Table 26.3 Morphological and biochemical classification of clostridia

Position of spores	Both proteolytic and saccharolytic		Saccharolytic	Neither proteolytic nor saccharolytic
Central or subterminal	**Proteolytic predominance** *C. bifermentans* *C. botulinum* A, B, F *C. histolyticum* *C. sordellii* *C. sporogenes*	**Saccharolytic predominance** *C. perfringens* *C. septicum* *C. chauvoei* *C. novyi*	*C. fallax* *C. botulinum* C, D, E *C. tertium* *C. tetanomorphum* *C. sphenoides*	
Oval and terminal		*C. difficile*		*C. cochlearium*
Spherical and terminal		*C. tetani*		

Pathogenicity

Clostridia produce disease mainly by toxin production.

Exotoxin Pathogenic clostridia produce powerful exotoxins which are responsible for the pathogenesis and disease, e.g., tetanus, botulism and gas gangrene.

Invasive toxin *C. perfringens*, besides being toxigenic, is also invasive and can spread along the tissues and even cause septicemia.

General Procedures for Laboratory Confirmation of Clostridial Infections

- The sensitivity to oxygen varies in different species. **Of greater importance than the absence of oxygen is the provision of a sufficiently low redox potential (Eh) in the medium.** Some (e.g., *C. novyi*) are exacting anaerobes and die on exposure to oxygen, while others (e.g., *C. histolyticum*) are aerotolerant and may even grow aerobically.
- Reducing substances such as unsaturated fatty acids, ascorbic acid, glutathione, cysteine, thioglycolic acid, alkaline glucose, sulphites or metallic iron can be added to the media to make them anaerobic. The optimum temperature for pathogenic clostridia is 37°C and the pH required for growth is 7–7.4.
- Growth is relatively slow on solid media. Colonial characteristics are variable. Some species are hemolytic on **blood agar**.
- **Robertson's cooked meat broth** contains unsaturated fatty acids which take up oxygen, the reaction being catalysed by hematin in the meat and the sulphydryl compounds. This reduces the oxidation/reduction (OR) potential. Growth is indicated by turbidity of the medium. Most species produce gas. The saccharolytic species turns meat pink, while proteolytic species turn it black, producing a foul odour.
- **Thioglycolate broth** is another liquid medium which supports the growth of clostridia.
- Production of acid, clot and gas can be detected in **litmus milk medium**.

CLOSTRIDIUM PERFRINGENS

Clinical Case

A 50-year-old man met with a road traffic accident. He sustained multiple fractures with open wounds and crush injury of the leg. He was taken to the nearest hospital two days later, at which time, he was found to be in shock. He was started on supportive therapy and antibiotics. There was edema and pain at the site with increased discolouration and a serous discharge. The area around the wound had crepitus on palpation. Microscopic examination of the wound discharge showed the presence of thick, brick-shaped, gram-positive bacilli along with gram-positive cocci. Based on a provisional diagnosis of gas gangrene, immediate surgical treatment with extensive excision of the local area (to prevent further spread) and intravenous penicillin with clindamycin were given. The exudate was also inoculated into Robertson's cooked meat medium. Clostridium perfringens and peptostreptococci grew in the culture.

C. perfringens (old nomenclature: *C. welchii, Bacillus aerogenes capsulatus, B. phlegmonis emphysematosae*) was originally cultivated by Achalme (1891) and first described in detail by Welch and Nuttall (1892). It is a normal inhabitant of the large intestines of humans and animals.

It is found in the feces and contaminates the skin of the perineum, buttocks and thighs. Its spores are commonly found in soil, dust and air.

This is the most common clostridia causing **gas gangrene**. It also causes food poisoning and necrotic enteritis in humans.

Spores

Spores are usually destroyed within five minutes by boiling. 'Food poisoning'-producing type A strains and certain type C strains resist boiling for 1–3 hours. **Autoclaving at 121°C for 15 minutes kills them**. Spores are resistant to commonly used antiseptics and disinfectants.

Pathogenicity

Virulence factors

C. perfringens strains are **classified into five types, A to E**, based on the toxins they produce. Typing is based on neutralisation tests with specific antitoxins by intracutaneous injections in guinea pigs or intravenous injection in mice.

Toxins

C. perfringens produces at least 12 distinct toxins, besides many other enzymes and biologically active soluble substances. The **four major toxins, alpha, beta, epsilon and iota**, are predominantly responsible for pathogenicity (Table 26.4).

Table 26.4 Toxins produced by *C. perfringens* types

Type	Pathogenicity	Major toxins				Minor toxins							
		α	β	ε	ι	γ	δ	η	θ	κ	λ	μ	ν
A	Gas gangrene: wound infections, septicemia	+++	–	–	–	–	–	±	±	±	–	±	±
	Food poisoning	+++	–	–	–	–	–	–	±	±	–	±	++
B	Lamb dysentery	+++	+++	++	–	+	±	–	++	–	+++	+++	++
C	Enteritis in animals	+++	+++	–	–	–	–	–	+++	+++	–	–	±
	Enteritis necroticans in humans	+++	++	–	–	++	–	–	–	–	–	–	+++
D	Enterotoxemia of sheep	+++	–	+++	–	–	–	–	++	++	++	±	±
E	Might be a pathogen of sheep and cattle	+++	–	–	+++	–	–	–	++	++	++	±	±

Major toxins

- **Alpha (α) toxin** is produced by all types of *C. perfringens* and most abundantly by type A strains. It is responsible for the profound toxemia of gas gangrene. It is **lethal, dermonecrotic and hemolytic**. It is a phospholipidase (**lecithinase C**) which, in the presence of Ca^{++} and Mg^{++} ions, splits lecithin into phosphoryl choline and diglyceride. **This reaction is seen as opalescence in serum or egg yolk media and is specifically neutralised by the antitoxin as described under Nagler's reaction.**
- **Beta (β), epsilon (ε)** and **iota (ι) toxins** have lethal and necrotising properties.

Minor toxins

- **Gamma (γ)** and **eta (η) toxins** have minor lethal action.
- **Delta (δ) toxin** has a lethal effect and is hemolytic for the red cells of even-toed ungulates (sheep, goats, pigs and cattle).
- **Theta (θ) toxin** is an oxygen-labile hemolysin, antigenically related to streptolysin O. It is also lethal and a general cytolytic toxin.
- **Kappa (κ) toxin** is a collagenase.
- **Lambda (λ) toxin** is a proteinase and gelatinase.
- **Mu (μ) toxin** is a hyaluronidase.
- **Nu (ν) toxin** is a deoxyribonuclease.

Enzymes

- Enzymes that destroy the blood group substance, A and H, also include **neuraminidase** (destroys myxovirus receptors on red blood cells), hemagglutinin (active against human RBC), fibrinolysin and hemolysin (distinct from the alpha, theta and delta toxins).

Two other enzymes associated with the pathogenesis of *C. perfringens* are:

- **Bursting factor,** which has a specific action on muscle tissue and is responsible for the characteristic muscle lesions in gas gangrene.
- **Circulating factor,** which can cause an increase in the adrenaline sensitivity of the capillary bed and also inhibit phagocytosis.

Clinical Manifestations

C. perfringens produces the following human infections:

1. Gas gangrene (also known as clostridial myonecrosis)

***C. perfringens* type A** is the predominant causative agent of gas gangrene. However, most commonly, they may lead only to wound contamination or anaerobic cellulitis. Gas gangrene (**anaerobic myositis)** results only when muscle tissues are invaded.

2. Food poisoning

Some strains of type A can produce food poisoning. They produce a **heat-labile enterotoxin** which, like the enterotoxins of *V. cholerae* and enterotoxigenic *E. coli*, leads to fluid accumulation in the rabbit ileal loop.

Food poisoning by *C. perfringens* is usually caused by a cold or warmed-up meat dish. When contaminated meat is cooked, the spores in the food may survive. During subsequent storage or warming, they germinate and multiply in the anaerobic environment of the cooked meat. Large numbers of clostridia may thus be consumed, which may pass into the intestines, unharmed by gastric acid due to the high protein content of the meat. **In the**

intestines, their spores produce the enterotoxin. After an incubation period of 8–24 hours, abdominal pain, diarrhea and vomiting set in.

Diagnosis Isolating heat-resistant *C. perfringens* type A from feces is not of much value on account of its presence in the normal intestine. Isolation from food has to be attempted by direct plating on selective media, as the bacillus is present in food mainly as vegetative cells.

Treatment The illness is self-limiting and recovery occurs in 24–48 hours.

3. Gangrenous appendicitis

C. perfringens **type A** (and **occasionally type D)** strains have been isolated from gangrenous appendicitis. It has been proposed that the toxemia and shock in some cases of intestinal obstruction and peritonitis may be due to the toxins of *C. perfringens*.

4. Necrotising enteritis

This is a severe and often fatal enteritis. It is caused by *C. perfringens* **type C** strains with heat-resistant spores which germinate in the intestine, producing beta toxin and causing mucosal necrosis.

Immunisation with the type C toxoid has been shown to protect against this condition.

> The name '**pigbel**' in New Guinea pidgin is abdominal pain and diarrhea following unaccustomed feasting on pig meat along with sweet potatoes, which contains trypsin inhibitors.

5. Other infections

- **Biliary tract infection**—acute emphysematous cholecystitis and post-cholecystectomy septicemia
- **Endogenous gas gangrene of intra-abdominal origin**—endogenous organism derived from the gut contaminates the abdominal wall during surgery
- **Brain abscess and meningitis** caused by *C. perfringens* have been reported very rarely
- **Panophthalmitis** following penetrating eye injuries
- **Thoracic infections** follow penetrating wounds of the thorax; they are often seen in battle casualties

- **Urogenital infections** following surgical procedures such as nephrectomy
- **Septic abortion**—infection of the uterus is a serious and not infrequent condition, commonly associated with septic abortion; septicemia is common in this condition

CLOSTRIDIUM SEPTICUM

This bacteria was first described by Pasteur and Joubert (1887) and called *Vibrion septique*. It is a pleomorphic bacillus, about 3–8 × 0.6 μm in size, forming oval, central or subterminal spores. It is motile due to the presence of peritrichate flagella. Colonies are initially irregular and transparent, turning opaque on continued incubation. Hemolysis occurs on horse blood agar. Growth is promoted by glucose. It is saccharolytic and produces abundant gas.

Virulence Factors

Six groups have been recognised, based on somatic and flagellar antigens.

C. septicum produces at least four distinct toxins. The **alpha toxin** is hemolytic, dermonecrotic and lethal; the **beta toxin** is a leucotoxic deoxyribonuclease, **the gamma toxin** a hyaluronidase and the **delta toxin** an oxygen-labile hemolysin. It produces a fibrinolysin.

C. septicum is found in the soil or in animal intestines and causes gas gangrene in association with other clostridia. It **also causes 'braxy' in sheep and 'malignant edema' in cattle and sheep**.

Treatment

Treatment of gas gangrene includes IV penicillin or clindamycin or metronidazole. Surgical debridement is done to remove dead and devitalised tissue. In addition to wound care, general supportive measures are required. Mortality is 100% in untreated cases. With rapid and appropriate treatment, mortality may be 50%.

CLOSTRIDIUM NOVYI (C. OEDEMATIENS)

This is a large, stout, pleomorphic gram-positive bacillus with oval,

subterminal spores. It is widely distributed in soil. It is a strict anaerobe, readily inactivated on exposure to air. Four types (A to D) are recognised, based on the production of toxins. **Only type A is of medical importance, as it causes gas gangrene.**

CLOSTRIDIUM HISTOLYTICUM

This is an actively proteolytic clostridium, forming oval, subterminal, bulging spores. It is aerotolerant, and some growth may occur even in aerobic cultures. It forms at least five distinct toxins. It is infrequently associated with **gas gangrene in humans**.

Gas Gangrene

Oakley (1954) defined gas gangrene as a rapidly spreading, **edematous myonecrosis**, occurring characteristically in association with severe crush injuries, leading to wounds of extensive muscle masses contaminated with pathogenic clostridia, particularly *C. perfringens*. The disease has been referred to in the past as malignant edema. Other descriptive terms are **anaerobic (clostridial) myositis** and **clostridial myonecrosis** (Fig. 26.3).

Fig. 26.3 Gas gangrene of the lower limb showing edema and discoloured skin

Polymicrobial Etiology

The bacteriology of gas gangrene is varied. Rarely is this due to infection by a single clostridium. Generally, several species of clostridia are found in association with anaerobic streptococci and facultative anaerobes such as *E. coli*, *Proteus* and staphylococci. Among the pathogenic clostridia, ***C. perfringens*** is the most frequently encountered (approximately 60 per cent), with ***C. novyi*** **and** ***C. septicum*** next (20–40 per cent) and ***C. histolyticum*** less often. Other clostridia usually found are *C. sporogenes*, *C. fallax*, *C. bifermentans, C. sordellii, C. aerofoetidum* and *C. tertium*. These organisms may not be pathogenic by themselves.

Pathogenesis

The infection may be exogenous or endogenous:

- **Exogenous** Clostridia usually enter a wound along with implanted foreign particles such as soil (particularly manured or cultivated soil), road dust, bits of clothing or shrapnel. They may also be present on normal skin, especially on the perineum and thighs.
- **Endogenous** Infection may be seen after clean surgical procedures (especially amputations for vascular disease) and even injections (especially adrenaline).

The condition favouring infection is low oxygen tension. The ionised calcium salts and silicic acid in the soil cause necrosis. Crushing of tissues or tearing of the arteries produces anoxia of the muscle. Extravasation of blood increases the pressure on the capillaries, further reducing blood supply. The Eh and pH of the damaged tissues fall, and these changes, along with chemical changes including the breakdown of carbohydrates and the liberation of amino acids from proteins, provide the ideal conditions for the proliferation of anaerobes. Extravasated hemoglobin and myohemoglobin are reduced and cease to act as oxygen carriers. As a result, aerobic oxidation is halted and anaerobic reduction of pyruvate to lactate leads to a further fall in Eh.

Mechanism of tissue damage

Clostridia multiply and elaborate toxins which cause further tissue damage as follows:

- Lecithinases damage cell membranes and increase capillary permeability, leading to extravasation and increased tension in the affected muscles, causing further anoxic damage.
- Lysis of erythrocytes by the alpha toxin causes hemolytic anemia and hemoglobinuria.
- Collagenases destroy collagen barriers in the tissues.
- Hyaluronidases break down the intercellular substances, causing invasive spread by the clostridia.
- Abundant production of gas reduces blood supply still further by pressure effects, extending the area of anoxic damage. It thus becomes possible for the infection to spread from the original site, making the lesion a progressive one.

Clinical Presentation

The incubation period may be as short as seven hours or as long as six weeks after the wound was created, the average being 10–48 hours with *C. perfringens*, 2–3 days with *C. septicum* and 5–6 days with *C. novyi* infection. Pain, tenderness and edema of the affected part along with systemic signs of toxemia are the cardinal features. There is a thin, watery discharge from the wound, which later becomes profuse and serosanguinous (Fig. 26.4). **Accumulation of gas makes the tissues crepitant** (*Clinical case*). In untreated cases, the disease process extends rapidly to profound toxemia and prostration, leading to death due to circulatory failure.

Fig. 26.4 Clostridial myonecrosis (*Source:* Public Health Image Library, ID 17603 / Dr. Robert Fass, Ohio State Dept. of Medicine / CDC)

Laboratory Diagnosis

The diagnosis of gas gangrene must be made primarily on clinical grounds; the function of the laboratory is only to provide confirmation of the clinical diagnosis and identification and enumeration of the infecting organisms. **The mere presence of clostridia in wounds does not constitute a diagnosis of gas gangrene.**

> Bacteriological examination also helps to differentiate gas gangrene from anaerobic streptococcal myositis, which may be indistinguishable from it clinically in the early stages. (In the latter, Gram-stained films show large numbers of streptococci and pus cells but not bacilli, contrasting with the scanty pus cells and diverse bacterial flora seen in films from gas gangrene.)

1. Specimens

- **Tissue bits** from the muscles at the edge of the affected area, from the necrotic area and from the exudate in the deeper parts of the wound are collected for investigations.
- **Exudates** from the parts where infection appears to be most active and from the depths of the wound may be collected with a

capillary pipette or a swab (which must be soaked in the exudate).

- **Blood cultures** may be required as they are often positive, especially in *C. perfringens* and *C. septicum* infections. However, *C. perfringens* bacteremia may occur without gas gangrene.

2. **Microscopic examination** Gram-stained films provide presumptive information about the species of clostridia. Large numbers of brick-shaped, gram-positive bacilli without spores is strongly suggestive of *C. perfringens* infection (Fig. 26.5). 'Citron bodies' and boat- or leaf-shaped pleomorphic bacilli with irregular staining suggest *C. septicum*. Large bacilli with oval, subterminal spores indicate *C. novyi*. Slender bacilli with round, terminal spores may be *C. tetani* or *C. tetanomorphum*.

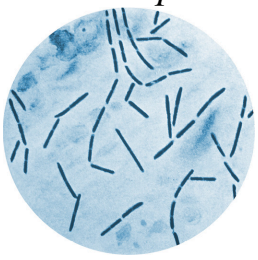


Fig. 26.5 Methylene blue-stained culture specimen revealing the presence of numerous *Clostridium septicum* bacteria:note the absence of spores Source: Public Health Image Library, ID 217 / CDC)

3. **Culture** Clostridia are anaerobes but can also grow under microaerophilic conditions. They grow within a pH range of 5.5–8.0 and temperature range of 20–50°C. Though usually grown at 37°C, a temperature of 45°C is optimal for many strains.

- **Robertson's cooked meat medium** This is the most commonly used medium for transport as well as culture. Subcultures from this transferred to blood agar plates yield pure or predominant growth of
  C. perfringens.
- **Fresh and heated blood agar** The colonies, on overnight incubation on rabbit, sheep or **human blood agar, show target hemolysis**, resulting from a narrow zone of complete hemolysis due to theta toxin and a much wider zone of incomplete

hemolysis due to the alpha toxin. This double zone pattern of hemolysis may fade on longer incubation.

- **Gram stain** shows plump, gram-positive bacillus with straight, parallel sides and rounded or truncated ends, about 4–6 × 1 μm in size. They are capsulated and non-motile. Spores are central or subterminal but are **rarely seen in artificial culture or in material from pathological lesions**, and their absence is one of the characteristic morphological features of *C. perfringens.*
- A plate of **serum or egg yolk agar** with *C. perfringens* antitoxin spread on one half is used for the **Nagler's reaction**. Figure 26.6 shows the toxin neutralised by the antitoxin so there is no opacity around the colonies. On the right, opacity is seen due to the presence of toxin which causes the opacity of the serum due to the breakdown of lecithin.

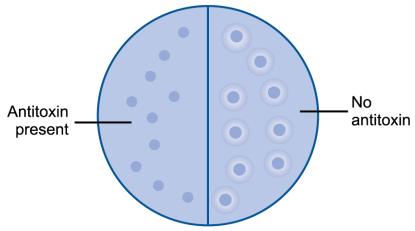


Fig. 26.6 Nagler's reaction

- **Nagler's reaction** When *C. perfringens* is grown on a medium containing 6% agar, 5% Fildes' peptic digest of sheep blood and 20% human serum, with the antitoxin spread on one half of the plate, colonies on the other half without the antitoxin will be surrounded by a zone of opacity. There will be no opacity around the colonies on the half of the plate with the antitoxin due to the specific neutralisation of the alpha toxin. This specific lecithinase effect, known as the **Nagler's reaction**, is a useful test for the rapid detection of *C. perfringens* in clinical specimens.

- The **reverse CAMP test** is used to identify *C. perfringens*; in this test, *C. perfringens* is streaked across the centre of the plate. *Streptococcus agalactiae* is streaked at right angles to it. A

positive reverse CAMP test shows the presence of an arrow-shaped zone of enhanced hemolysis pointing towards *C. perfringens* (Fig. 26.7).

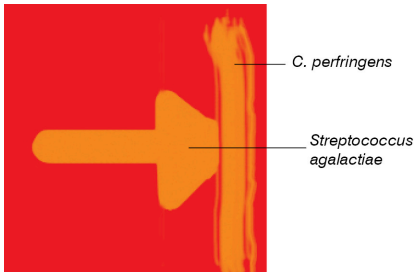


Fig. 26.7 Reverse CAMP test

- In **litmus milk**, fermentation of lactose leads to the formation of acid, which is indicated by the change in colour of the litmus from blue to red. The acid coagulates the casein (acid clot) and the clotted milk is disrupted due to vigorous gas production. This is known as **stormy clot formation**.
- **Hot–cold lysis** Alpha toxin is hemolytic for red cells due to its action on the phospholipids on the erythrocyte membranes. Lysis is of the hot–cold variety, best seen after incubation at 37°C followed by cooling at 4°C.

4. **Biochemical reactions** Glucose, maltose, lactose and sucrose are fermented with the production of acid and gas. Clostridia are indole-negative, MR-positive and VP-negative. H_2S is formed abundantly. Most strains reduce nitrates.

Prophylaxis and Treatment

- **Surgery** is an essential prophylactic and therapeutic measure in gas gangrene. All damaged tissue should be removed promptly and the wounds irrigated to remove blood clots, necrotic tissue and foreign materials. In established gas gangrene, uncompromising excision of all affected parts may be life-saving. Where facilities exist, **hyperbaric oxygen** may be beneficial in treatment.

- **Antibiotics** are effective in prophylaxis, in combination with surgical methods. The drug of choice is **metronidazole given intravenously** along with clindamycin and penicillin before surgery and repeated every eight hours for 24 hours.
- **Passive immunisation** with 'anti–gas gangrene serum' (equine polyvalent antitoxin in a dose of 10,000 IU *C. perfringens*, 10,000 IU *C. novyi* and 5,000 IU *C. septicum* antitoxin given IM or in emergencies, IV) is the common practice in prophylaxis.

CLOSTRIDIUM TETANI

C. tetani is the causative organism of tetanus. Tetanus has been known since very early times, having been described by Hippocrates and Aretaeus. Kitasato (1889) isolated it in pure culture and reproduced the disease in animals by inoculation of pure cultures.

C. tetani is widely distributed in soil and in the intestines of humans and animals. It is ubiquitous and has been recovered from a wide variety of other sources, including street and hospital dust, cotton wool, plaster of Paris, bandages, catgut, talc, wall plaster and clothing.

Morphology

It is a gram-positive, slender bacillus, about 4–8 × 0.5 ∝m in size, though there may be considerable variation in length. The spores are spherical, terminal and bulging, giving the bacillus the characteristic '**drumstick**' **appearance**
(Fig. 26.8). It is non-capsulated and motile due to the presence of peritrichate flagella.
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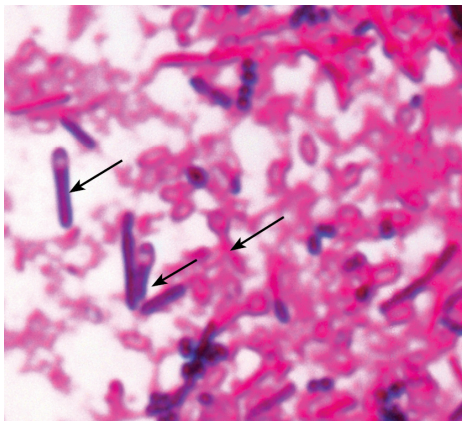


Fig. 26.8 *C. tetani*: drumstick appearance on Gram stain (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Serotypes

Ten serological types have been recognised based on agglutination (types I to X).

Pathogenicity

C. tetani has little invasive power. The toxin produced locally is absorbed by the motor nerve endings and transported to the central nervous system intra-axonally. The toxin is specifically and avidly fixed by gangliosides of the grey matter of the nervous tissue.

Toxins

C. tetani produces at least two distinct toxins—**a hemolysin (tetanolysin)** and a powerful **neurotoxin (tetanospasmin).** The two are antigenically and pharmacologically distinct and their production is mutually independent. **A third toxin, a non-spasmogenic, peripherally active neurotoxin, has been identified. It is not known whether this plays any role in the pathogenesis of tetanus.**

- ***Tetanospasmin*** This is the toxin responsible for tetanus. It is oxygen-stable but relatively heat-labile, being inactivated at 65°C in five minutes. It is plasmid-coded.

  Mechanism of action Tetanus neurotoxin, tetanospasmin, binds to the presynaptic membrane of the neuromuscular junction. Then, it is internalised and transported retroaxonally to the spinal cord.

It blocks the release of neurotransmitters (glycine and GABA – gamma-aminobutyric acid) from the spinal inhibitory interneuron, resulting in spastic paralysis, induced by the toxin. Tetanospasmin resembles **strychnine** in its effects. The toxin acts **pre-synaptically**. The abolition of spinal inhibition causes uncontrolled spread of impulses initiated anywhere in the central nervous system. This results in muscle rigidity and spasms due to the simultaneous contraction of agonists and antagonists in the absence of reciprocal inhibition.

Lethal dose The purified toxin is active in extremely small amounts and has a minimum lethal dose (MLD) of about 50–75 $\times 10^{-6}$ mg in mice.

Tetanospasmin gets toxoided spontaneously or in the presence of low concentrations of formaldehyde. It is a good antigen and is specifically neutralised by the antitoxin. It is a simple protein composed of a single polypeptide chain. On being released from the bacillus, it autolyses to form a heterodimer consisting of a heavy chain (93,000 MW) and a light chain (52,000 MW) joined by a disulphide bond. The tetanus and botulinum toxins resemble each other in their amino acid sequences.

- ***Tetanolysin*** This is a heat-labile, oxygen-labile hemolysin, antigenically related to the oxygen-labile hemolysins of *C. perfringens*, *C. novyi* and *S. pyogenes*. It is not relevant in the pathogenesis of tetanus.

Resistance

Spores can survive in soil for years. The heat resistance of tetanus spores appears to be subject to strain differences. Most are killed by boiling for 10–15 minutes but some resist boiling for up to three hours. Autoclaving at 121°C for 20 minutes is recommended to kill the spores.

Disinfectants such as iodine (1% aqueous solution) and hydrogen peroxide (10 volumes) kill the spores within a few hours. They are not destroyed by 5% phenol or 0.1% mercuric chloride solution.

Tetanus

Tetanus is characterised by **tonic muscular spasms** with painful muscle rigidity. Pain often begins in the jaw muscles (**trismus or 'lockjaw')** and neck, shoulder and abdominal muscles. As the disease progresses, muscle groups throughout the body are affected and spontaneous generalised seizure-like **tetanospasms** develop. Autonomic dysfunction, including hypertension and tachycardia alternating with bradycardia occurs. In the absence of ventilatory support, death is usually due to respiratory failure.

The disease follows entry of the spores into sterile body sites following injury (puncture wounds), surgical operations (due to lapse in asepsis), unhygienic practices (septic abortion) and unsterile injections. The incidence of tetanus has come down greatly due to immunisation and infection control practices. **It is not transmitted from person to person**.

Incubation period This is variable, ranging from two days to several weeks, but is commonly 6–12 days.

Mortality Untreated tetanus has a very high mortality rate, notably neonatal tetanus.

Neonatal tetanus It develops by the 8th day of life. The child becomes irritable, refuses feeds and shows facial grimace with stiff muscles (Fig. 26.9). In rural India, tetanus used to be a common cause of death, particularly in the newborn. However, universal immunisation of infants and expectant mothers has reduced the incidence of maternal and neonatal tetanus to a large extent.

Fig. 26.9 Neonatal tetanus caused by *C. tetani* showing muscle stiffness (*Source:* Public Health Image Library, ID 6374 / CDC)

Laboratory Diagnosis

1. **Cultural characteristics** The diagnosis of tetanus should always be made on clinical grounds. Laboratory tests are not usually helpful. Laboratory diagnosis may be made by the demonstration of *C. tetani* by microscopy, culture or toxigenicity tests.

2. **Specimen** Necrotic tissue may be collected from the site of injury.

3. **Microscopy** It is unreliable; the demonstration of the typical **'drumstick'** bacilli in wounds in itself is not diagnostic of tetanus. The bacilli may be present in some wounds without tetanus developing. It may be indistinguishable from *C. tetanomorphum* and *C. sphenoides*.

4. **Culture** *C. tetani* is an obligatory anaerobe that grows only in the absence of oxygen. The optimum temperature is 37°C and pH 7.4. It grows on ordinary media. Growth is improved by blood and serum but not by glucose. Isolation is more likely from excised bits of tissue from the necrotic depths of wounds than from wound swabs.

Swarming
C. tetani produces **swarming growth.** An extremely fine, translucent film of growth is produced, which is practically invisible, except at the delicately filamentous advancing edge. The incorporation of polymyxin B, to which other clostridia are resistant, makes the medium selective for *C. tetani*.

- **Fildes technique** If the water of condensation at the bottom of a nutrient agar slope is inoculated with the mixed bacterial culture, a pure growth of *C. tetani* will be obtained from the upper part of the medium after 24 hours of anaerobic incubation.
- **Deep agar shake cultures** The colonies appear as spherical fluffy balls, 1–3 mm in diameter, made up of filaments with a radial arrangement. In **gelatin stab cultures**, a fir tree type of growth occurs, with slow liquefaction. *C. tetani* grows well in **Robertson's cooked meat broth**, with turbidity and some gas formation. The meat is not digested but is turned black on prolonged incubation. On **blood agar**, α-hemolysis is produced, which later develops into β-hemolysis due to the production of hemolysin (**tetanolysin**).

5. **Toxigenicity testing**

- **In vitro** Inhibition of hemolysis on blood agar incorporated with tetanus antitoxin (1,500 units/mL) denotes neutralisation of hemolysin (tetanolysin) in contrast to hemolytic colonies on blood agar without the antitoxin.
- **In vivo** Toxigenicity can also be tested in animals. A 2–4-day-old cooked meat culture (0.2 mL) is inoculated into the root of the

tail of a mouse. A second mouse that has received a dose of tetanus antitoxin (1,000 units) an hour earlier serves as the control. In 12–24 hours, the test mouse's tail begins to show stiffness. Rigidity proceeds to the leg on the inoculated side, the opposite leg, trunk and forelimbs, in that order. The animal dies within two days.

Prophylaxis, Treatment and Prevention

The nature of prophylaxis depends largely on the type of the wound and the immune status of the patient.

If the vaccination status is not known or has been >5 years ago, an intramuscular injection of tetanus toxoid is advised immediately after a penetrating injury or laceration.

Treatment with antibiotics

- Metronidazole, 500 mg, every six hours intravenously or orally depending on the extent of infection is recommended.
- Penicillin G (100,000–200,000 IU/kg/day intravenously), is given in 2–4 divided doses.
- Tetracyclines, macrolides, clindamycin, cephalosporins and chloramphenicol are also effective.
- **Bacitracin** or **neomycin** may be applied locally.
- **Human tetanus immunoglobulin (or anti-tetanus immunoglobulin) (TIG)** 10,000 IU, suitably diluted, may be given by slow IV infusion, followed, if needed, by another 5,000 IU later. Even though TIG may not neutralise the toxin already bound to the nervous tissue, it can inactivate the unbound toxin and any further toxin that may be produced.

Patients recovering from tetanus should receive a full course of **active immunisation with tetanus toxoid**, as an infection does not confer subsequent immunity. Second attacks of tetanus have been recorded.

Surgical management This aims at removing foreign bodies, necrotic tissue and blood clots in order to remove any anaerobic environment

favourable for *C. tetani*. The extent of surgical treatment varies from simple cleansing to radical excision, depending on the type of the wound.

Supportive management Control of spasms is done by benzodiazepine, and autonomic dysfunctions by magnesium sulphate. Airway and respiratory control is mandatory.

Prevention by immunisation The most reliable method of prevention is to induce active immunity by universal immunisation of children with booster doses when appropriate.

- **Passive immunisation** was originally done with **Anti-Tetanus Serum (ATS)** from hyperimmune horses. The drawbacks of ATS are **immune elimination** and **hypersensitivity**. Prior sensitisation also leads to type III hypersensitivity reactions, which may range from mild local reactions to serum sickness. Infrequently, fatal anaphylaxis (type I hypersensitivity) may also occur**. It is compulsory that a test for hypersensitivity be done prior to the administration of ATS**.

  Tetanus immunoglobulin (TIG) provides passive immunity without the risk of hypersensitivity. This is effective in smaller doses (250 units) and has a longer half-life (3–5 weeks). **Passive immunisation is an emergency procedure to be used only once.**
- **Active immunisation** is the most effective method of prophylaxis. This is achieved by spaced injections of:
  - **Formol toxoid,** which is available as '**plain toxoid**' or
  - **Adsorbed toxoid,** which is adsorbed on aluminium hydroxide or phosphate. The tetanus toxoid is given alone or with the diphtheria toxoid and the pertussis vaccine as the '**triple vaccine**', in which the pertussis vaccine acts as an adjuvant also.

  Dosage A course of immunisation consists of three doses of tetanus toxoid given intramuscularly, with an interval of 4–6 weeks between the first two injections and the third dose given six months later (or according to the recommendations of the National Immunisation Programme). A full course of immunisation **confers immunity for a**

period of at least ten years. A '**booster dose**' of toxoid is recommended after ten years. A booster dose of toxoid is given if penetrating injury occurs three years or more after the full course of immunisation. **ATS or TIG should not be given to an immunised individual.**

- **Combined immunisation** consists of administering to a non-immune person exposed to the risk of tetanus, a TIG injection at one site, along with the first dose of toxoid at the contralateral site, followed by the second and third doses of toxoid at monthly intervals. Ideally, combined immunisation should be used whenever passive immunisation is called for.

Table 26.5 shows the recommended integrated prophylaxis of tetanus following injury.

Table 26.5 Tetanus prophylaxis in the wounded

Nature of wound	Immune status of the person		
	Immune	**Partially immune**	**Non-immune**
Clean (wound toilet performed within six hours)	Toxoid × 1*	Toxoid × 1	Toxoid × 3
Contaminated (soil or other for eign or necrotic material present)	Toxoid × 1*	Toxoid × 1TIG Antibiotics	Toxoid × 3 TIG Antibiotics
Infected	Toxoid × 1* Antibiotics	Toxoid × 1TIG Antibiotics	Toxoid × 3 TIG Antibiotics

Note:
Immune—Patient has had a full course of three injections of toxoid
Partially immune—Patient has had two injections of toxoid
Non-immune—Patient has had one or no injection of toxoid, or immunisation status is not known
TIG—Tetanus immunoglobulin
* The toxoid needs to be given only if three years or more have elapsed after active immunisation or the last booster injection.

Maternal and Neonatal Tetanus (MNT)

According to the WHO, maternal and neonatal tetanus are the most common life-threatening consequences of unclean deliveries and umbilical cord care practices. Mortality rates are extremely high, especially when appropriate medical care is not available, and is seen in low-income countries. This had been an indicator of inadequate access to immunisation and other maternal, newborn, and child health services. However, with immunisation of pregnant women becoming mandatory, these rates have greatly come down in India.

CLOSTRIDIUM BOTULINUM

C. botulinum causes **botulism**, a paralytic disease that usually presents in the form of food poisoning. The name botulism is derived from the Latin word *botulus* meaning 'sausage'. *C. botulinum* was first isolated by van Ermengem (1896) from a piece of ham that caused an outbreak of botulism.

Morphology

C. botulinum is motile due to the presence of peritrichate flagella and produces subterminal, oval and bulging spores.

Spores

Spores are heat- and radiation-resistant, surviving several hours at 100°C and for up to 10 minutes at 120°C. Spores of the non-proteolytic types B, E and F are much less resistant to heat.

Classification

Eight types of *C. botulinum* strains have been identified **(A, B, C1, C2, D, E, F** and **G)** based on the immunological differences. They have similar pharmacological activity but are neutralised only by the homologous antiserum. C2 toxin shows enterotoxic activity, while the others are neurotoxins.

Pathogenicity

Pathogenicity of *C. botulinum* is due to the action of its toxin, the

manifestations of which are collectively called **botulism**.

Toxin

The potent toxin produced by *C. botulinum* differs from other exotoxins in that it is not released during the life of the organism. It is produced intracellularly and appears in the medium only on cell death and autolysis. It is believed to be synthesised initially as a non-toxic protoxin or progenitor toxin. Trypsin and other proteolytic enzymes activate the progenitor toxin to produce active toxin.

The toxin has been isolated as a pure crystalline protein, which is probably the most toxic substance known. It has a MW of 70,000, and the lethal dose for mice is 0.000,000,033 mg. The lethal dose for human beings is probably 1–2 mg. It is a neurotoxin and acts slowly, taking several hours to kill. **It is one of the most potent toxins known to humankind.** Toxin production appears to be determined by the presence of bacteriophages, at least in types C and D.

The toxin is relatively stable and is inactivated only after 30–40 minutes at 80°C or after 10 minutes at 100°C. Food suspected to be contaminated with the botulinum toxin can be rendered completely safe by pressure cooking or boiling for 20 minutes.

Mechanism of action It acts by **blocking the production or release of acetylcholine at the synapses and neuromuscular junctions**. Onset is marked by diplopia, dysphagia and dysarthria due to cranial nerve involvement. A symmetric descending paralysis is the characteristic pattern, ending in death by respiratory paralysis.

Clinical uses of toxins

A small quantity of *C. botulinum* type A toxin injected into a muscle selectively weakens it by blocking the release of acetylcholine at the neuromuscular junction. Muscles atrophy but recover in 2–4 months as new terminal axons form and restore transmission. Intramuscular injection of the toxin was first used to treat strabismus. It is now recognised as a safe and effective symptomatic therapy for many neuromuscular diseases and is used cosmetically to temporarily remove wrinkles and frown lines.

Botulism

Clinical types

Food-borne botulism This is caused by the ingestion of pre-formed toxin.

Human disease is usually caused by types A, B, E and very rarely, F. Types C and D are usually associated with outbreaks in cattle and wild fowl. Type G has been associated with sudden death in a few patients. The source of botulism is usually preserved food—meat and meat products. Type E is associated with fish and other seafood. Cans of spoiled food are often inflated and show bubbles on opening. Non-proteolytic varieties leave food unchanged. Symptoms usually begin 12–36 hours after ingestion of food. Coma or delirium may supervene. No vomiting or diarrhea is present. Death is due to respiratory failure and occurs 1–7 days after onset. Case fatality varies at 25–70 per cent.

Wound botulism This is a very rare condition resulting from wound infection with *C. botulinum*. Type A has been responsible for most of the cases studied.

Infant botulism *C. botulinum* spores ingested in baby food get established in the gut and produce the toxin. These infections occur in infants below six months who are bottle-fed. The manifestations are constipation, poor feeding, lethargy, weakness, pooled oral secretions, weak or altered cry, floppiness, flaccid paralysis, ptosis and loss of head control (Fig. 26.10). Some cases of sudden infant death syndrome have been attributed to infant botulism. Honey used to sweeten milk or formula food has been incriminated as a likely food item through which the bacillus enters the gut.

Management consists of supportive care and assisted feeding. **Antitoxins and antibiotics are not indicated.**

Fig. 26.10 Infant botulism—flaccid facial musculature and ptosis of the lids caused by *Clostridium botulinum*. (*Source:* Public Health Image Library, ID 16681 / Dr. V.R. Dowell, Jr. / CDC)

Laboratory Diagnosis

1. **Microscopy** Diagnosis may be confirmed by the demonstration of the bacillus or the toxin in food or feces. Gram-positive spore-forming bacilli may be demonstrated in smears made from the food. *C. botulinum* may be isolated from the food or the patient's feces.

2. **Animal inoculation** The food is macerated in sterile saline, and the filtrate inoculated into mice or guinea pigs intraperitoneally. Control animals protected by polyvalent antitoxin remain healthy.

3. **Cultural characteristics** It is a strict anaerobe. Optimum temperature is 35°C but some strains may grow even at 1–5°C. Colonies are large, irregular and semitransparent with a fimbriate border.

Prevention and Treatment

As most cases of botulism follow the consumption of inadequately canned or preserved food, control can be achieved by **proper canning and preservation.**

When an **outbreak** occurs, a **prophylactic dose of antitoxin** should be given intramuscularly to all who consumed the food article.

Active immunisation has been shown to be effective. Two doses of aluminium sulphate adsorbed toxoid are given at an interval of ten weeks, followed by a booster a year later. Polyvalent antiserum to types A, B and E may be administered as soon as a clinical diagnosis is made.

Supportive therapy with maintenance of respiration is of great importance.

CLOSTRIDIUM DIFFICILE

C. difficile is the most common cause of health care-associated diarrhea in many developed countries following the use of broad-spectrum antibiotics like clindamycin, ampicillin or fluoroquinolones to which the organism is resistant.

The clinical entity is known as **pseudomembranous colitis** which is associated with acute colitis with bloody or watery diarrhea.

Pathogenesis

Two high-molecular-weight **exotoxins, A and B**, are involved in the pathogenesis of the condition. Toxin A is a potent enterotoxin which

attaches to gut receptors; it may also be cytotoxic. Toxin B is a cytotoxin. **Toxin genes are present on a chromosomal pathogenicity island**.

Diagnosis

- Direct examination by colonoscopy shows microabscesses. **Culture** of stool specimens on cycloserine, cefoxitin fructose agar (CCFA), or cysteine, cefoxitin yeast extract agar (CCYA) selective media are used to recover *C. difficile*. This is important for typing for epidemiological purposes.
- **Detection of toxins A and/or B in stool by ELISA is the mainstay of diagnosis.**
- The toxin can be demonstrated in the feces of patients by its characteristic effect on Hep-2 or/and human diploid cell cultures.
- The toxin is specifically neutralised by the *C. sordellii* antitoxin.
- **Molecular methods** have been employed to detect genes for toxin A or B.
- Detection of toxin by lateral flow assay has been used as a rapid screening test to detect *C. difficile* toxins and glutamate dehydrogenase from stool samples.

Prevention and Treatment

The disease is prevented by **restricting the use of antibiotics associated with *C. difficile* outbreaks.**

The condition is treated by discontinuing the antibiotic causing the disease and instituting **vancomycin or metronidazole**.

Fecal transplant

Recently, interest has been generated in the treatment of ulcerative colitis with fecal transplants. Fecal transplantation (or bacteriotherapy) is the transfer of stool from a healthy donor into the gastrointestinal tract of another for restoring their normal flora. This is done to treat recurrent *C. difficile* colitis, which is associated with prolonged antibiotic therapy. Some success has been noted. Further studies need to be done to expand the role of fecal transplants in restoring normal flora.

Antibiotic-associated diarrhea

This is seen in patients on prolonged antibiotic use and is mainly due to the alteration of the normal flora. About 20–25% of cases are due to *C. difficile* but other anaerobic infections are also implicated in the causation.

Key points

- Members of the genus *Clostridium* are gram-positive, rod-shaped bacteria which usually exhibit motility and are largely spore-forming, obligatory anaerobes. Clostridia are naturally found in soil and water, animal and human excreta and animal products.
- *Clostridium botulinum* causes botulism (food poisoning), infant botulism and wound botulism.
- In infant botulism, bacterial spores that have contaminated honey used to sweeten cow's milk or formula germinate in the child's gut.
- *Clostridium perfringens* (*Clostridium welchii*) causes gas gangrene and clostridial food poisoning.
    - In gas gangrene, spores from the soil contaminate open wounds in which spores may germinate if there is an anaerobic environment and produce toxins.
    - In food poisoning, spores in contaminated meat germinate following mild heating; if this occurs in an anaerobic environment (packaged foods), organisms grow rapidly, producing enterotoxin.
    - *C. perfringens* are gram-positive bacilli with subterminal spores.
    - On blood agar, organisms cause a double zone of hemolysis.
- *Clostridium tetani* causes tetanus (lockjaw). Spores in the soil are introduced through a puncture wound deep in the tissues. The anaerobic environment of the deep tissues allows the spores to germinate, and the bacilli release the tetanus toxin–tetanospasmin.
- Tetanus can be prevented by active and passive immunisation.
- *Clostridium difficile* causes antibiotic-associated diarrhea and pseudomembranous colitis.
- *C. difficile* toxins A and/or B can be detected from stools by enzyme immunoassays. The disease is treated with vancomycin or metronidazole.

Questions

Essays

1. Explain the pathogenesis and laboratory diagnosis of gas gangrene.
2. Describe the pathogenesis and laboratory diagnosis of tetanus and outline the prophylactic measures.

Short Answers

1. Antibiotic-associated diarrhea
2. Food poisoning by *Clostridium perfringens*
3. Pathogenesis of gas gangrene
4. Pathogenesis of tetanus
5. Virulence factors of *C. perfringens*
6. Virulence factors of *C. tetani*

7. Types and pathogenesis of botulism

Part III: Bacteriology

CHAPTER 27

Non-sporing Anaerobic Bacteria

Chapter navigation

- Anaerobic cocci
- Anaerobic bacilli

INTRODUCTION

Non-sporing anaerobic bacteria differ widely in the degree of anaerobiosis required for their growth. Some species fail to grow if the atmosphere contains as little as 0.03% oxygen (obligatory), while others are aerotolerant and may grow sparsely on the surface of aerobic plates (facultative). Consequently, the techniques employed for the propagation and study of anaerobes vary in complexity. Several anaerobes exist in soil and water and may be of industrial and agricultural importance (e.g., *Methanobacteria* and *Butyrivibrios*). DNA base composition and analysis of fatty acid end products of metabolism have been used to differentiate the anaerobes.

ANAEROBIC COCCI

Anaerobic cocci represent a heterogeneous collection of cocci and can be divided into the gram-positive and gram-negative groups.

Gram-Positive Cocci

Peptostreptococcus

These are relatively small cocci (0.2–2.5 µm). Many of them are aerotolerant and grow well under 10% CO_2 in an aerobic atmosphere.

They are **normal inhabitants of the vagina, intestines and mouth**. They may cause several clinical infections such as puerperal sepsis and other

genital infections, wound infections, gangrenous appendicitis, urinary tract infections, osteomyelitis and abscesses in the brain, lungs and other internal organs. They are often seen in large numbers in pus from suppurative lesions, so a Gram-stained smear may be helpful in diagnosis. Infections are usually polymicrobial. *Peptostreptococcus anaerobius* is most often responsible for puerperal sepsis and
P. magnus for abscesses. *P. asaccharolyticus*, *P. tetradius* and *P. prevotii* are some other species commonly present in clinical specimens.

Gram-Negative Cocci

Veillonella

These are gram-negative cocci of varying sizes occurring as diplococci, short chains or groups. They are normal inhabitants of the mouth, and of the intestinal and genital tracts. *Veillonella parvula* has been reported from clinical specimens but its pathogenic role is uncertain.

All anaerobic cocci are generally sensitive to penicillin, chloramphenicol and metronidazole, and resistant to streptomycin and gentamicin.

NON-SPORING ANAEROBIC GRAM-POSITIVE BACILLI

This group contains several genera, of which the medically relevant are *Eubacterium, Propionibacterium, Lactobacillus, Mobiluncus* and *Bifidobacterium*.

- *Lactobacillus* is present in the mouth, intestines and, typically, in the adult vagina (**Doderlein's bacilli**). It is generally non-pathogenic, though *L. catenaforme* has been associated with bronchopulmonary infections.
- *Bifidobacterium* is a pleomorphic rod that shows true and false branching. It is present in large numbers in the intestines and in the mouth.
- *Mobiluncus* species are motile, curved, anaerobic bacilli that may appear as gram-variable rods.
  M. mulieris and *M. curtisii* have been isolated from the vagina in **bacterial vaginosis**, along with ***Gardnerella vaginalis*.**

Bacterial vaginosis is a polymicrobial infection characterised by a thin, malodorous vaginal discharge. Its '**rotten fish**' **smell** is accentuated when it is mixed with a drop of KOH solution. The vaginal pH is more than 4.5. **Clue cells** (epithelial cells whose surface is covered by adherent bacilli) are seen in stained or unstained films. Diagnosis is made by **Nugent's score** which is based on the morphotypes of bacteria seen on examining the Gram-stained smear of the vaginal discharge.

ANAEROBIC GRAM-NEGATIVE BACILLI

Clinical Case

A 65-year-old woman who has been a diabetic for several years, presented to neurosurgery OPD with symptoms of focal seizures. Computed tomography of the brain showed a 'space-occupying' lesion in the left cerebral hemisphere with 'ring-enhancement', which was consistent with brain abscess. The pus was sent in a sealed gassed-out vial filled with carbon dioxide. Microscopic examination showed the presence of gram-positive cocci and a few fine gram-negative bacilli. Culture on anaerobic blood agar incubated in GasPak was positive for peptostreptococci and Bacteroides fragilis.
A surgical drainage was done, and she was treated with cefotaxime and metronidazole, to which she responded.

Medically important anaerobic gram-negative bacilli belong to the family Bacteroidaceae and are classified into the genera *Bacteroides, Porphyromonas*, *Prevotella, Fusobacterium* and *Leptotrichia*.

Bacteroides

These are non-motile, strict anaerobes, usually pleomorphic, appearing as slender rods, branching forms or coccobacilli, seen singly, in pairs or in short chains. They grow well on media such as brain–heart infusion agar in an anaerobic atmosphere containing 10% CO_2. They possess **capsular polysaccharides which are the virulence factors**. They evoke an antibody response that can be detected in patients. *Bacteroides* are normal inhabitants of the intestinal, respiratory and female genital tracts. They are susceptible to metronidazole, clindamycin and chloramphenicol. *B. melaninogenicus* is susceptible to penicillin, while *B. fragilis* is not.

B. fragilis is the anaerobe most frequently isolated from clinical specimens. It is often recovered from blood, pleural and peritoneal fluids, CSF, brain abscesses (*Clinical case*), wounds and urogenital infections.

Porphyromonas

Previously classified under *Bacteroides*, this is a saccharolytic pigmented species that has been separated as the genus *Porphyromonas*, containing *P.*

gingivalis—responsible for periodontal disease and *P. endodontalis*—causing dental root canal infections.

Prevotella

Previously classified under *Bacteroides*, the moderately saccharolytic species inhibited by 20% bile has been placed in the genus *Prevotella*, containing *P. melaninogenica,*
P. buccalis, P. denticola and others.

P. melaninogenica is easy to recognise because of the black or brown colour of its colonies (Fig. 27.1)

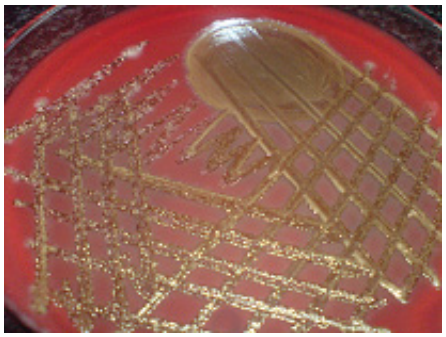


Fig. 27.1 *P. melaninogenica* on anaerobic blood agar

The colour is not due to the melanin pigment, as was once thought, but due to a hemin derivative. It has been isolated from various infections including lung or liver abscess, mastoiditis, intestinal lesions and lesions of the mouth and gums. Cultures of *P. melaninogenica* and even dressings from wounds infected with the bacillus **produce a characteristic red fluorescence when exposed to ultraviolet light**.

Fusobacterium

This contains long, thin or spindle-shaped bacilli with pointed ends. *F. nucleatum* is a normal inhabitant of the mouth and is found in oral infection and pleuropulmonary sepsis. *F. necrophorum* produces a wide range of exotoxins and has been responsible for liver abscess and other abdominal infections in animals and less often, in humans. ***Leptotrichia* contains a single species, *L. buccalis,* which was formerly known as Vincent's fusiform bacillus** or ***Fusobacterium fusiforme***. It is a long, straight or slightly curved rod, often with pointed ends. It is part of the normal oral flora and is seen in acute necrotising lesions in the mouth. A common

condition is Vincent's angina, which may resemble diphtheria, with the inflamed pharyngeal mucosa showing a greyish membrane which peels easily. Stained smears show large fusiform and spiral Borrelia bacilli.

ANAEROBIC INFECTIONS

Anaerobic infections are usually endogenous and are caused by tissue invasion by bacteria normally resident on the respective body surfaces. Anaerobic bacteria are normally present on the skin, mouth, nasopharynx and upper respiratory tract, intestines and vagina (Table 27.1).

Table 27.1 Normal anaerobic flora of the human body

Anaerobe	Skin	Mouth–nasopharynx	Intestine	Vagina
Clostridium			++	
Actinomyces		+		
Bifidobacterium		+	++	+
Propionibac-terium	++			
Bacteroides fragilis			++	
P. melaninogenica		++	+	++
Fusobacterium		++	+	
Gram-positive cocci		++	++	++
Gram-negative cocci		++	+	++
Spirochetes		+		

Characteristics of anaerobic infections

- Anaerobic infections generally follow some **precipitating factor** such as trauma, tissue necrosis, impaired circulation, hematoma formation or the presence of foreign bodies.
- Diabetes, malnutrition, malignancy or prolonged treatment with aminoglycoside antibiotics may act as **predisposing factors**.
- They are typically **polymicrobial**, more than one anaerobe being responsible besides aerobic bacteria (*Clinical case*).
- While the infection is usually localised, general **dissemination** may occur by bacteremia.

- Some **clinical features** suggest the presence of anaerobic infection.
- **Pus** produced by anaerobes is characteristically putrid, with a pervasive, nauseating odour. However, there may be exceptions; infections solely due to *B. fragilis* may be free of this smell.
- Pronounced **cellulitis** is a common feature of anaerobic wound infections.
- Toxemia and fever are not marked.

Table 27.2 lists the common sites and types of anaerobic infections and the bacteria responsible.

Table 27.2 Common anaerobic infections and the causative bacteria

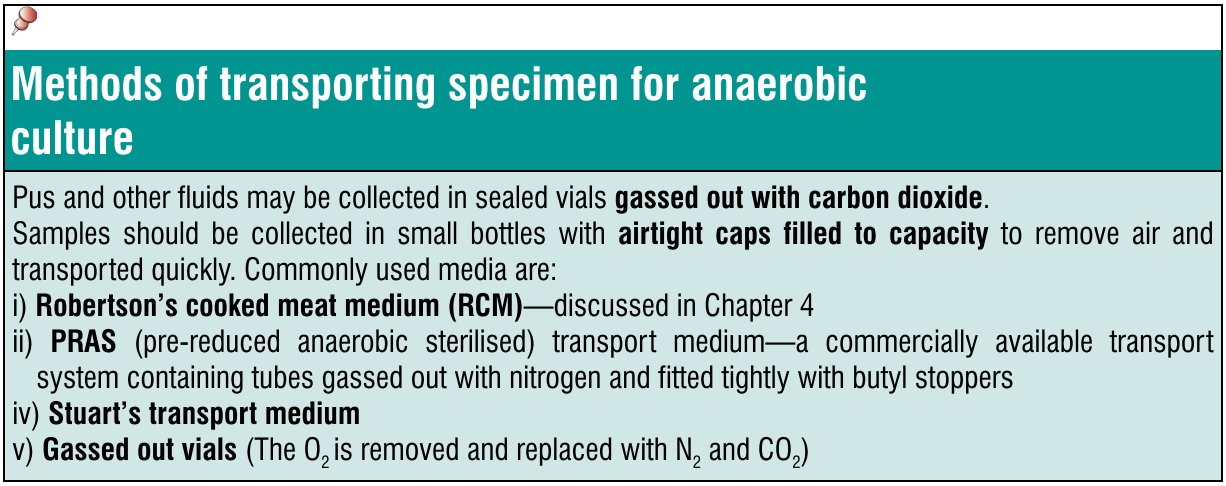
Site and type of infection	Causative bacteria
Central nervous system: Brain abscess	*B. fragilis; Peptostreptococcus*
Ear, nose, throat: Chronic sinusitis, otitis media, mastoiditis, orbital cellulitis	Fusobacteria (aerobes frequently responsible)
Mouth and jaw: Ulcerative gingivitis (Vincent's)	Fusobacteria, spirochetes
Dental abscess, cellulitis	Mouth anaerobes
Abscess and sinus of jaw	*Actinomyces*, other mouth anaerobes
Respiratory: Aspiration pneumonia, lung abscess, bronchiectasis, empyema	Fusobacteria, *P. melaninogenica*, anaerobic cocci; *B. fragilis* rarely
Abdominal: Subphrenic, hepatic abscess; appendicitis, peritonitis; ischiorectal abscess; wound infection after colorectal surgery	*B. fragilis*
Female genitalia: Wound infection following genital surgery; puerperal sepsis; tubo-ovarian abscess	*P. melaninogenica,* anaerobic cocci; *B. fragilis*
Bartholin's abscess, septic abortion	Genital anaerobes and *C. perfringens*
Skin and soft tissue: Infected sebaceous cyst	Anaerobic cocci
Breast abscess, axillary abscess	Anaerobic cocci; *P. melaninogenica* (*S. aureus* commonest cause)
Cellulitis, diabetic ulcer, gangrene	*B. fragilis* and others

Laboratory Diagnosis

1. **Specimen** As anaerobes form part of the normal flora of the skin and mucous surface, their isolation from specimens has to be interpreted

cautiously. The mere presence of an anaerobe does not prove its causal role. Specimens should be collected in such a manner as to avoid resident flora. For example, **sputum is unsatisfactory for culture in a suspected case of lung abscess**; the ideal specimen in such a case is the material collected by aspiration. Wound swabs are generally unsatisfactory but where they are to be used, they should be sent in a transport medium.

2. **Transport The key to anaerobic culture is an oxygen-free environment.** As some anaerobes die on exposure to oxygen, care should be exercised to minimise contact with air during collection, transport and handling of specimens. Transport and culture are done using different methods to ensure anaerobic atmosphere during transport.



Methods of transporting specimen for anaerobic culture

Pus and other fluids may be collected in sealed vials **gassed out with carbon dioxide**.
Samples should be collected in small bottles with **airtight caps filled to capacity** to remove air and transported quickly. Commonly used media are:
i) **Robertson's cooked meat medium (RCM)**—discussed in Chapter 4
ii) **PRAS** (pre-reduced anaerobic sterilised) transport medium—a commercially available transport system containing tubes gassed out with nitrogen and fitted tightly with butyl stoppers
iv) **Stuart's transport medium**
v) **Gassed out vials** (The O_2 is removed and replaced with N_2 and CO_2)

3. **Microscopy** In the laboratory, exposure should be kept to the minimum. Examination of a Gram-stained smear is useful. Pus in anaerobic infection usually shows a large variety of different organisms and numerous pus cells. Rarely, as in brain abscess, only one type of organism may be seen.

4. **Ultraviolet examination** Examination of the specimen under ultraviolet light may show **the bright red fluorescence of *P. melaninogenica***.

5. **Gas–liquid chromatography** This procedure may yield presumptive information from the specimen on the types of anaerobes present.

6. **Culture** Several special media have been described for anaerobes.

i) Freshly prepared **blood agar with neomycin, yeast extract, hemin and vitamin K** is adequate for routine diagnostic work. Plates are incubated at 37°C in an anaerobic jar with 10% CO_2. Parallel aerobic cultures should always be set up. This is necessary as a control for

growth on anaerobic plates and also because, in most anaerobic infections, aerobic bacteria are also involved.

ii) The **Gaspak system** provides a convenient method for routine anaerobic cultures. Plates are examined after 24 or 48 hours. Some anaerobes, such as fusobacteria, require longer periods of incubation. Automated BacT/ALERT or BACTEC may be used to isolate anaerobes.

Treatment

Penicillin, clindamycin and metronidazole are the most active drugs against anaerobes. The exception is the *Bacteroides* species where resistance to penicillin and clindamycin have been reported. Amoxicillin/clavulanic acid or ampicillin/sulbactam combinations or carbapenems can be given. In the case of abscess, surgical drainage has to be carried out along with antibiotics.

Key points

- Many anaerobic bacteria cause disease in human beings but the majority are normal commensals.
- Anaerobic cocci represent a heterogeneous collection of cocci broadly divided into the gram-positive and gram-negative groups.
- The anaerobic gram-positive *Peptostreptococcus* is a normal inhabitant of the vagina, intestines and mouth.
- Anaerobic gram-positive bacilli include *Eubacterium, Propionibacterium*, *Lactobacillus*, *Bifidobacterium* and *Mobiluncus*. *Propionibacterium acnes* is present on the skin and has been implicated in late postoperative endophthalmitis. *Lactobacillus* is present in the mouth, intestines and, typically, in the adult vagina; it is generally non-pathogenic. The *Mobiluncus* species are motile, curved anaerobic bacilli that may appear as gram-variable rods; they have been isolated from the vagina in bacterial vaginosis, along with *Gardnerella vaginalis*.
- Medically important anaerobic gram-negative bacilli of this family include the *Bacteroides* and *Fusobacterium* species.
- Transport of clinical specimens and cultures requires special conditions and media so that the specimen is not exposed to oxygen.

Questions

Essays

1. Describe the transport and culture of clinical samples for anaerobes.
2. Describe the laboratory diagnosis of anaerobic infections.

Short Answers

1. Bacteroides
2. Clue cells

Part III: Bacteriology

Chapter 28

Mycobacteria I

Chapter navigation

- *Mycobacterium tuberculosis*
- Pulmonary tuberculosis
- Pathogenesis
- Current methods of diagnosis
- Drug resistance
- Elimination programme

INTRODUCTION

Mycobacteria are slender rods that do not stain readily but once stained, resist decolourisation with dilute mineral acids due to the presence of mycolic acid in their cell walls. They are called **acid-fast bacilli** (AFB). The genus *Mycobacterium* contains three groups: **obligate parasites**, **opportunistic pathogens** and **saprophytes** (Table 28.1). In liquid cultures, they form a mould-like pellicle (hence the name mycobacteria, meaning fungus-like bacteria). Mycobacteria are slow-growing, aerobic, non-motile, non-capsulated and non-sporing bacteria.

Table 28.1 Some common mycobacteria and their habitats

Obligate parasites	*M. tuberculosis,* *M. bovis, M. leprae*	Species are always considered pathogens
Opportunistic pathogens	*M. scrofulaceum*, *M. kansasii,* *M. marinum,* *M. ulcerans,* *M. avium-intercellulare* complex (MAC)	Uncommon causes of human disease Infect immuno-compromised individuals
Saprophytes	*M. smegmatis,* *M. gordonae,* *M. flavescens*	Found in soil and water; cause environmental contamination

Obligate Parasites

***Mycobacterium tuberculosis* complex** Koch (1882) isolated the mammalian tubercle bacillus and proved its causative role in tuberculosis by satisfying four basic principles of infectious agents (known as **Koch's postulates;** see Chapter 1). The complex consists of:

- *Mycobacterium tuberculosis*
- *M. bovis*
- *M. africanum,* which causes human tuberculosis in tropical Africa
- Other non-human pathogens—*M. caprae* (cattle pathogen), *M. microti* (voles and other small mammals), *M. canetti* (similar to *M. africanum*) and *M. pinnipedii* (pathogen of seals)

Mycobacterium leprae The second human pathogenic mycobacterium is the lepra bacillus, which causes leprosy. It was discovered by Hansen in 1868.

Opportunistic Pathogens

Non-tuberculous mycobacteria (NTM) These include a heterogeneous group of organisms that infect birds and other cold-blooded and warm-blooded animals. They have been isolated from skin ulcers in patients as well as from soil, water and other environmental sources. They are broadly categorised as **photochromogens, scotochromogens, non-photochromogens and rapid growers,** based on their growth rates and pigmentation in the presence or absence of light. They are opportunistic pathogens, infecting immunocompromised individuals.

Saprophytes

Saprophytic mycobacteria These include *M. phlei* from grass and *M. smegmatis* (a rapidly growing mycobacteria seldom found in the smegma) which frequently contaminates urine cultures.

MYCOBACTERIUM TUBERCULOSIS

Morphology

M. tuberculosis is a straight or slightly curved rod, about 3 ∝m × 0.3 ∝m in size. Its size depends on conditions of growth. Long, filamentous, club-shaped and branching forms may sometimes be seen. *M. bovis* is usually straighter, shorter and stouter.

Staining Characteristics of Mycobacteria

Acid-fast staining

Mycobacteria, when stained with strong carbol fuchsin by the Ziehl–Neelsen method, resist decolourisation by 20% sulphuric acid and are therefore called acid-fast. Acid-fastness has been ascribed to the **presence of an unsaponifiable lipid-rich (mycolic acid)** material in the cell wall or to a semipermeable membrane around the cell.

Staining may be uniform or granular. Beaded or barred forms are frequently seen in *M. tuberculosis*, whereas *M. bovis* stains more uniformly. They are seen as slender pink bacilli in a smear (Fig. 28.1).

Electron micrographs of thin sections show a thick cell wall composed of three layers enclosing a trilaminar plasma membrane. They transform into spheroplasts and L-forms when grown in the presence of lysozymes.

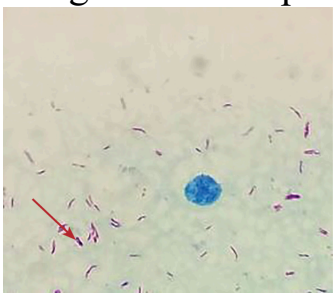


Fig. 28.1 *Mycobacterium tuberculosis*: thin, pink coloured bacilli in sputum smear (acid-fast bacilli on ZN stain) (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Alcohol-fastness

In addition to being acid-fast, *M. tuberculosis* is also **alcohol-fast** (resists decolourisation with 3% hydrochloric acid in 95% alcohol), which differentiates it from saprophytic mycobacteria, which are only acid-fast.

Kinyoun's modification of acid-fast staining

This is a modified cold method. It requires increasing the concentration of phenol acid and duration of staining. The smear is decolourised with 20%

sulphuric acid followed by decolourisation with 95% ethanol for two minutes. The two steps can be combined using acid alcohol (3% HCl in 95% ethanol). Loeffler's methylene blue, 1% picric acid or 0.2% malachite green are used as counterstain. Under the oil immersion objective, the acid-fast bacilli are seen as bright red rods while the background is blue, yellow or green depending on the counterstain used.

Fluorochrome staining

When several smears are to be examined daily, it is convenient to use fluorescence microscopy. Smears are stained with auramine phenol or auramine rhodamine fluorochrome dyes and examined under ultraviolet illumination or LED (light-emitting diode). Bacilli appear as bright rods against a dark background. Because of the contrast, the bacilli can be seen even under the high dry objective, enabling large areas of the smear to be screened rapidly.

Differences between *M. tuberculosis* and saprophytic mycobacteria on staining

Microscopic demonstration of acid-fast bacilli provides only presumptive evidence of tuberculosis, as even saprophytic mycobacteria may present a similar appearance. Saprophytic mycobacteria stain uniformly without a barred or beaded appearance, and are usually only acid-fast. These mycobacteria may be present in the environment and tap water, and can contaminate clinical materials. Saprophytes may pose problems with gastric aspirates, feces and urogenital specimens.

Antigenic Properties

Several antigens have been identified in the mycobacterial cell wall and the cytoplasm.

1. The **cell wall** contains three major antigens which are responsible for the virulence of the organism:
   i) ***Lipids*** The mycobacterial cell wall is rich in long-chain fatty acids called **mycolic acids**. Mycolic acids, when complexed with peptidoglycan, are responsible for granuloma formation. **The pattern of lipids on gas chromatography has been used to classify**

different species. Another factor called the **cord factor is also responsible for the virulence** of bacteria.

ii) ***Lipoarabinomannan*** It is a cell wall-associated glycolipid and a virulence factor that can inhibit macrophage activation, leading to the persistence of mycobacteria within mononuclear phagocytes.

iii) ***Proteins*** These induce delayed-type hypersensitivity and elicit tuberculin reaction. There is also some antigenic relationship between lepra and tubercle bacilli.

iv) ***Polysaccharides*** Group specificity is due to polysaccharides. Their role in pathogenesis is not clear but they can induce immediate-type hypersensitivity.

Antibodies against the polysaccharide, protein and phosphatide antigens of tubercle bacilli have no protective or diagnostic relevance.

2. **Cytoplasmic antigens** are protein antigens that stimulate dendritic cells and increase the secretion of cytokines.

Host Range

M. tuberculosis causes natural infection in humans, other primates, and other animals that have close contact with humans. Experimentally, guinea pigs and hamsters are highly susceptible to the infection (mice are only moderately susceptible).

M. bovis produces tuberculosis in cattle, humans, other primates, carnivores including dogs and cats, badgers, swine, parrots and some birds of prey. Experimentally, guinea pigs are highly susceptible to this pathogen while rats are moderately susceptible. Bacillus Calmette–Guerin (BCG), the tuberculous vaccine, is an attenuated strain of *M. bovis*.

Resistance

Mycobacteria are killed at 60°C in 15–20 minutes. Bacilli in sputum may be viable for 20–30 hours and in droplet nuclei, up to 8–10 days under suitable conditions. Cultures remain viable at room temperature for 6–8 months and may be stored for up to two years at –20°C.

Susceptibility of mycobacteria to commonly used disinfectants in the hospital

Mycobacteria can survive exposure to 5% phenol, 15% sulphuric acid, 3% nitric acid, 5% oxalic acid and 4% sodium hydroxide. They are killed by formaldehyde and glutaraldehyde. They are destroyed by tincture of iodine in five minutes and by 80% ethanol in 2–10 minutes. Ethanol is a suitable disinfectant for skin, gloves and clinical thermometers.

TUBERCULOSIS

Clinical Case 1

A 12-year-old boy complained of low-grade fever and mild headache for the previous two weeks, which worsened over the last two days. He also had vomiting, confusion and stiffness in the neck, for which he was brought to the outpatient department. His father was a known case of pulmonary TB but had defaulted on treatment. CSF examination showed a mild increase in cell counts with predominant lymphocytes. Proteins were raised and glucose was low. Gram stain and ZN of centrifuged sample of CSF did not reveal any organisms. PCR assay targeting the unique sequence of M. tuberculosis in the CSF was positive. Two weeks later, the culture using the BACTEC-MGIT system was positive for M. tuberculosis. A drug susceptibility by line probe assay (LPA) showed that the strain was resistant to rifampicin and INH (isoniazid). The patient was treated with second-line drugs, ofloxacin, PAS, cycloserine and ethionamide.

Clinical Case 2

A 50-year-old man presented with a history of low-grade fever with an evening rise in temperature and productive cough for the past two months. More recently, he had expectorated blood-tinged sputum. His also gave a history of loss of weight (around 10 kg over the previous four months) and loss of appetite. A chest X-ray revealed a nodular infiltrate in the apical area of the right upper lobe. The sputum smear was positive for AFB and culture of the same yielded M. tuberculosis. He was started on DOTS which included isoniazid, rifampicin, ethambutol and pyrazinamide daily for two months.

Tuberculosis is a potentially fatal infection caused mainly by *M. tuberculosis* complex (MTC), which can affect any part of the body, with the lungs being the most common organ involved.

Source

It is transmitted from person to person by aerosol produced by coughing, the source being an active case of tuberculosis. The aerosol may contain as many as 3,000 infectious nuclei per cough. The disease spreads most often among members of the household or close and prolonged contacts of open cases (whose sputum may contain a minimum

of 10,000 bacilli per mL). Infection also occurs infrequently by ingestion, through contaminated milk, and rarely, by inoculation.

The inhaled bacilli reach the lungs and are phagocytosed by the alveolar macrophages. This is known as the **Trojan Horse phenomenon,** wherein they are transported across the alveolar barrier by the infected alveolar macrophages. They escape being killed by inhibiting the phagosomal vacuole, escape from the vacuole to the cytosol and modulate macrophage apoptosis. Additionally, evidence is now emerging of an alternate mechanism. The bacteria, which are few and quiescent during initial infection, transform into a highly replicative and invasive phenotype. They directly migrate across the alveolar barrier into the circulation. This results in dissemination from the lung in primary infection. Several factors including the number and virulence of the infecting bacilli, host factors including genetic susceptibility, age, immunocompetence, stress, nutrition and co-existing illness influence the outcome of the infection. Spread of the disease can occur by:

- Erosion of the bronchus and deposition of the caseous matter, thus resulting in its spread to other parts of the lung.
- Dissemination via the bloodstream to other parts of the body.

Immunology

Humans are evidently able to mount an effective defence against the infection as only about a tenth of those infected develop active tuberculosis. **Cell-mediated immunity** is the specific immune mechanism that plays a major role in tuberculosis. Humoral immunity has little or no role in protection or pathogenesis. The **key cell is the activated $CD4^+$ helper T cell,** which can develop along two different paths:

i) T_h1 releasing cytokines such as interferon γ (gamma), interleukins 1 and 2, toxic effects of tumour necrosis factor α (TNF-α) and others exerting different biological effects. T_h1-dependent cytokines activate macrophages, resulting in protective immunity and containment of the infection.

ii) T_h2 cytokines induce delayed-type hypersensitivity (DTH), tissue destruction and progressive disease.

Allergy and immunity Infection with the tubercle bacillus induces cell-mediated immunity which manifests as delayed hypersensitivity (allergy) and resistance to infection (immunity).

Koch's phenomenon
This is of historical interest. Robert Koch demonstrated that when virulent tubercle bacilli are injected into a healthy guinea pig, it develops a nodule at the site of inoculation, which develops into an ulcer that persists, with caseation of draining lymph nodes. On the other hand, if the bacilli are injected into an already infected guinea pig (infected 4–6 weeks earlier), the lesion develops rapidly, within one or two days, into a shallow ulcer which heals completely without involving the draining lymph nodes.

Purified Protein Derivative (PPD) (Old Tuberculin OT) This was originally prepared by Robert Koch, and modified by Seibert. Subsequently, highly purified preparations of PPD have been developed, which are currently in use. A positive test is evidence that one is infected with *M. tuberculosis* but does not necessarily indicate active disease.

Pathogenesis

The essential pathology of primary tuberculosis is the Ghon focus in the lower lung. In post-primary tuberculosis, it is the production of a characteristic lesion, the **tubercle,** in infected tissues (Fig. 28.2). This is an avascular **granuloma** composed of a central zone containing giant cells, with or without caseation, and a peripheral zone of lymphocytes and fibroblasts.

Depending on the time of infection and the type of response, tuberculosis may be classified as primary and post-primary.

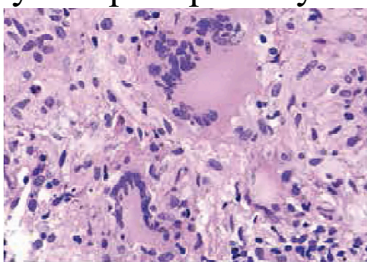


Fig. 28.2 Tubercle or granuloma with caseation

Primary tuberculosis is the initial infection by the mycobacteria in a host. In endemic countries like India, young children usually are more

susceptible. Alveolar macrophages engulf the bacilli, which multiply intracellularly. They give rise to a subpleural focus of tuberculous pneumonia, commonly located in the lower lobe or the lower part of the upper lobe (**Ghon focus**). The hilar lymph nodes are involved. The Ghon focus together with the enlarged hilar lymph node constitutes the **primary complex**. In most cases, the lesion heals spontaneously in 2–6 months, leaving behind a calcified nodule. In children with impaired immunity or other risk factors, the primary lesion may enlarge and cause miliary, meningeal or other forms of disseminated tuberculosis (*Clinical case* 1—meningeal tuberculosis).

Post-primary (secondary or adult) tuberculosis is due to the reactivation of a latent infection (post-primary progression, endogenous reactivation) or exogenous re-infection and differs from the primary type in many respects. **It affects mainly the upper lobes of the lungs, the lesions undergoing necrosis and tissue destruction, leading to cavitation** (Fig. 28.3). Lymph node involvement is unusual. The necrotic materials break out into the airways, leading to expectoration of bacteria-laden sputum, which is the main source of infection to contacts. In the immunodeficient, cavity formation is unusual. Instead, there is widespread dissemination of lesions in the lungs and other organs (*Clinical case* 2—pulmonary tuberculosis). The post-primary lesion heals with scarring (named Simons's focus after Georg Simon, a German pediatrician).

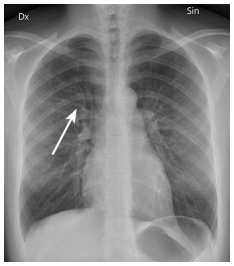


Fig. 28.3 Chest radiograph showing upper lobe cavity (*Source:* Wikipedia)

Tuberculous lesions are primarily of two types:

- **Exudative type** is an acute inflammatory reaction with accumulation of edema fluid, polymorphonuclear leucocytes, and later, of lymphocytes and mononuclear cells. This is typically seen when there are plenty of virulent bacilli and the host response is DTH rather than protective immunity.

- **Productive type** of lesion is predominantly cellular and associated with protective immunity.

Extrapulmonary tuberculosis (EPTB)

This is generally associated with HIV, other immunocompromised conditions and diabetes. Extrapulmonary tuberculosis (EPTB) comprises conditions caused by *Mycobacterium tuberculosis* in organs or tissues outside the lungs. There are many forms of EPTB, affecting every organ system in the body. They include TB meningitis, pleural TB and TB pericarditis, which are life-threatening. Others include spinal TB, renal TB and TB of the abdomen and pelvic organs, which cause significant ill-health and lasting disability.

Epidemiology

It is estimated that a third of the world's population (two billion), is infected with the tubercle bacilli. Every year, between eight and nine million new cases of tuberculosis appear, and three million persons die from the disease. The large majority of the cases of infections and deaths are from the less economically developed nations. India is one of the worst affected countries. More than 40 per cent of the population is infected and around 15 million suffer from tuberculosis. Over three million of these are highly infectious, open cases. Half a million people die from the disease every year in India—almost one person every minute.

Factors responsible for spread of tuberculosis

- **Poverty** and tuberculosis go hand in hand. Tuberculosis has declined rapidly in the developed nations due to an improvement in the standard of living but continues unabated in the underdeveloped countries.
- Currently, with the AIDS pandemic, tuberculosis has become a problem for developed nations as well, with outbreaks among the HIV-infected individuals. **A close relationship has emerged between tuberculosis and HIV.** Not only does HIV infection reactivate latent tuberculosis, it also makes the disease more serious and renders treatment ineffective. Tuberculosis may, in

turn, hasten the development of HIV infection into active disease.

- A third factor that has made the situation more grave is the emergence and spread of **multiple drug resistance** among tubercle bacilli. The serious threat of tuberculosis combined with multidrug resistance and concomitant HIV infection made the World Health Organization declare tuberculosis a global emergency in as early as 1993.

Human infection with *M. bovis* has become rare due to pasteurisation or boiling of milk prior to consumption. Person-to-person transmission of *M. bovis* is very rare.

Laboratory Diagnosis of Tuberculosis

Laboratory confirmation of tuberculosis is made by any one or more of the following, details of which are shown in the flowchart in Fig. 28.4 (updated in 2016 in accordance with the Revised National Tuberculosis Control Programme):

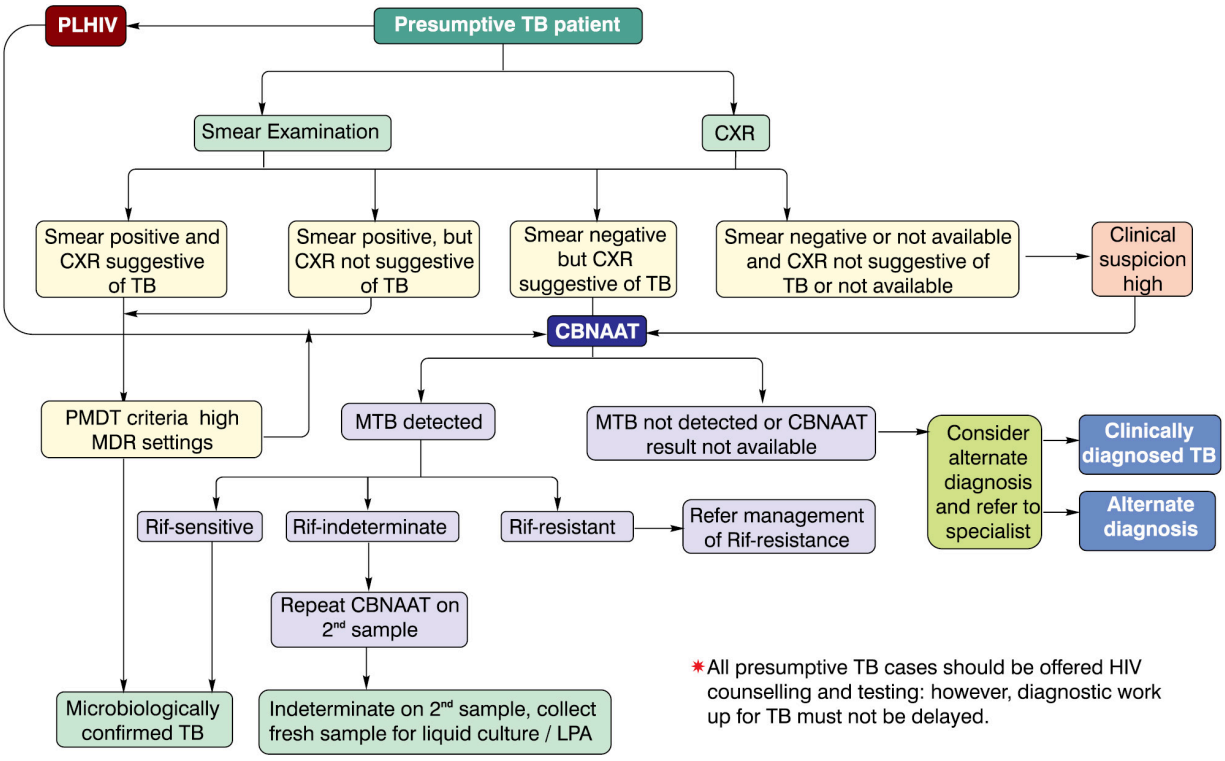

PLHIV
Presumptive TB patient
Smear Examination
CXR
Smear positive and CXR suggestive of TB
Smear positive, but CXR not suggestive of TB
Smear negative but CXR suggestive of TB
Smear negative or not available and CXR not suggestive of TB or not available
Clinical suspicion high
CBNAAT
PMDT criteria high MDR settings
MTB detected
MTB not detected or CBNAAT result not available
Consider alternate diagnosis and refer to specialist
Clinically diagnosed TB
Alternate diagnosis
Rif-sensitive
Rif-indeterminate
Rif-resistant
Refer management of Rif-resistance
Repeat CBNAAT on 2nd sample
Microbiologically confirmed TB
Indeterminate on 2nd sample, collect fresh sample for liquid culture / LPA
*All presumptive TB cases should be offered HIV counselling and testing: however, diagnostic work up for TB must not be delayed.

Fig. 28.4 Algorithm (flowchart) for identification of pulmonary tuberculosis (*Source:*

https://image.slidesharecdn.com/revised national tuberculosis control programme-161221132832/95/ revised-national-tuberculosis-control-programme- 30-638.jpg?cb=1482327017)

- Demonstrating the bacilli in the lesion by microscopy
- Isolating the bacilli in culture
- Using molecular diagnostic methods to detect DNA or RNA of the bacilli from clinical specimen

Demonstrating hypersensitivity to Purified Protein Derivative (PPD), otherwise known as Mantoux tuberculin skin test, is **useful to elicit prior exposure but not active disease.**

Pulmonary Tuberculosis

Specimen for diagnosis of pulmonary tuberculosis

Sputum Bacillary shedding in the sputum is abundant in caseation but relatively scanty in organised lesions that do not communicate with the airways. Sputum is best collected in the morning before any meal. Repeat testing of samples increases the chance of detection. Two sputum sample examinations have now been recommended by the Revised National TB Control Programme (RNTCP). Spot sample examination at the time of the first visit has been recommended. Spot sample refers to the sample collected during the patient's first visit to the health care setting. The second sample is an early morning one collected before any meal. **(CDC and WHO, however, recommend 3 sputum smear examinations.)**

If sputum is not available, **laryngeal aspirates** or **bronchial washings (with appropriate precautions)** may be collected.

In small children who tend to swallow their sputum, **gastric lavage** can be examined.

All laboratory procedures are mandated to be performed in biosafety cabinets Class II B. The use of personal protective gear like masks and gloves is mandatory. In the disposal of laboratory waste, biomedical waste guidelines have to be adhered to.

Decontamination and concentration of specimens

Specimens from non-sterile sites and sputum need prior treatment so that

organisms other than mycobacteria are inhibited. **Therefore, liquefaction, decontamination and concentration improve the yield**. All such methods should be done in Class II biosafety cabinets.

- ***Petroff's method*** Sputum is incubated with an equal volume of 4% sodium hydroxide solution at 37°C with frequent shaking till it becomes clear (on an average, this takes 20 minutes). It is then centrifuged at 3,000 rpm for 20 minutes and the sediment neutralised with N/10 HCl and used for smear. Excessive exposure to alkali is deleterious and should be avoided.
- ***NALC (N-acetylcysteine) combined with 2% NaOH*** This method is considered better than Petroff's. Here, **N-acetylcysteine** is used for the liquefaction of sputum. NaOH kills the contaminating bacteria. The sample is then neutralised with a buffer and concentrated by centrifugation. This method is also compatible with culture in automated systems.

Microscopy

Sputum microscopy is the single most reliable method in the diagnosis and control of tuberculosis. Smears should be prepared from the thick, purulent part of the sputum.
Routinely used staining methods are:

- **Ziehl–Neelsen technique (ZN)** Direct or concentrated smears of sputum are examined for AFB. Slender pink bacilli in a blue background are seen if the smear is positive.
- **Kinyoun's modification of acid-fast staining** Acid-fast bacilli are seen as bright pink rods while the background is blue, yellow or green, depending on the counterstain.

  A minimum of 10,000 acid-fast bacilli should be present per mL of sputum for them to be readily demonstrable in direct smears**.**

  At least 100 fields have to be examined by Ziehl–Neelsen technique over 10 minutes before giving a negative report; the report is said to be positive if two or more typical bacilli are detected. Smears are graded based on the RNTCP guidelines (Table 28.2).

Table 28.2 ZN smear evaluation for sputum smear as per RNTCP guidelines

Number of bacilli	RNTCP	
	Result	Grading (Report)
>10/field	+ve	3+
1–10/field	+ve	2+
10–99/100 field	+ve	1+
1–9/100 field	+ve	Scanty*
No. of AFB in 100	−ve	Negative
*Record actual no. of bacilli seen in 100 fields		

- **Auramine rhodamine** Bacilli appear as bright rods against a dark background.

Culture

Culture is the gold standard for diagnosis of tuberculosis, detecting as few as 10 to 100 bacilli per mL. *Mycobacterium tuberculosis* has a generation time of 14–15 hours, hence it grows slowly; **colonies appear between two weeks to eight weeks**. Optimum temperature is 37°C. Temperatures below 25°C or above 40°C do not favour growth. Optimum pH is 6.4–7.0. *M. tuberculosis* is an obligate aerobe.

Several media, both solid and liquid, are used for the cultivation of tubercle bacilli:

- ***Solid media*** Löwenstein–Jensen (LJ) medium is the most widely employed conventional media for routine culture. It consists of coagulated hen's eggs, mineral salt solution, asparagine and malachite green, the last acting as a selective agent that inhibits other bacteria (Fig. 28.5). The culture is incubated for up to 8 weeks and examined weekly for growth. Buff-coloured colonies, which are rough in consistency and not easily emulsified, are subjected to acid-fast staining to identify acid-fast bacilli.

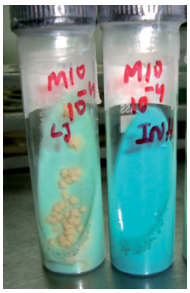


Fig. 28.5 *M. tuberculosis* on LJ medium (left); LJ medium without growth (right)

- ***Liquid media*** Among the several liquid media described, **Dubos', Middlebrook's, Proskauer and Beck's, Sula's and Sauton's** are the more commonly used ones. Virulent strains tend to form long, serpentine cords in liquid media**.**

Newer methods of cultivation

Several **automated culture methods** have been introduced to detect early growth and are slowly replacing culture methods.

- **BacT/ALERT** This method uses colourimetry for growth detection, which is measured on the basis of the production of CO_2 as a result of bacterial metabolism during growth.
- **BACTEC MGIT** This is an automated **mycobacteria growth indicator tube (MGIT)** (Fig. 28.6**)**. It is a rapid growth detection method, which uses 7H9 Middlebrook medium with fluorometric detection technology of O_2 consumption. An added advantage is that the incorporation of pyrazinamide (PZA) in the medium detects the organism's resistance to this drug.

Fig. 28.6 BACTEC MGIT for culture of *M. tuberculosis* (*Source:* Intermediate Reference Laboratory for TB, SCB Medical College, Cuttack, Odisha)

- **VersaTrek System** This is a continuous monitoring system for detecting the growth of mycobacteria.
  It detects pressure changes above the level of the medium either due to gas consumption or gas liberation resulting from bacterial growth. Recently, this has been improved to detect drug susceptibility to mycobacteria.

The two species which are required to be commonly differentiated in the laboratory are *M. tuberculosis* and *M. bovis* (Table 28.3).

Table 28.3 Differentiating characteristics of *M. tuberculosis* and *M. bovis*

Test	*M. tuberculosis*	*M. bovis*
Niacin	+	–
Nitrate	+	–
Catalase	+	–
Oxygen preference	Aerobic	Microaerophilic
Growth in T2H [Thiophene-2-carboxylic acid hydrazide (TCH)]	+	–
0.5% glycerol	Helps growth	No effect
Sodium pyruvate	Helps growth	Helps growth
p-nitrobenzoic acid (PNB)	No growth	No effect
Colony character	Dry, rough, raised, irregular, wrinkled	Flat, smooth, moist, white; break up easily

	surface; not emulsifiable	
Pyrazinamidase test	+	–
Host	Human	Bovine
Type of culture	Eugonic	Dysgonic

Growth on medium containing *p*-nitrobenzoic acid (PNB) Identification of *M. tuberculosis* is confirmed by the absence of growth on LJ medium containing PNB. This helps to differentiate *M. tuberculosis* complex from non-tuberculous mycobacteria.

Identification of *M. tuberculosis* depends on a combination of one or more tests together with growth at 25°C on Löwenstein–Jensen (LJ) medium and growth at 37°C on LJ containing *p*-nitrobenzoic acid (PNB). Other tests used in identification are niacin, catalase and nitrate tests.

Biochemical reactions

Several biochemical tests have been described for the identification of the mycobacterial species:

1. ***Niacin test*** Human tubercle bacilli form niacin when grown on an egg medium. 10% cyanogen bromide and 4% aniline in 96% ethanol turn the suspension of *M. tuberculosis* to canary-yellow (*M. tuberculosis* is positive and *M. bovis* is negative).
2. ***Aryl sulphatase test*** Only atypical mycobacteria give a positive result.
3. ***Catalase–peroxidase tests*** These help in differentiating tubercle bacilli (positive) from atypical mycobacteria and indicate sensitivity of the strain to isoniazid. **Catalase and peroxidase activities are lost when the tubercle bacilli become INH-resistant**.
4. ***Nitrate reduction test*** This is positive with *M. tuberculosis* and negative with *M. bovis* (Fig. 28.7).

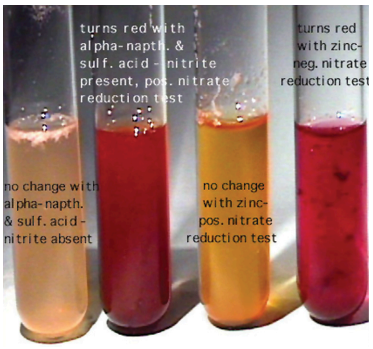

turns red with alpha-napth. & sulf. acid - nitrite present, pos. nitrate reduction test
turns red with zinc- neg. nitrate reduction test
no change with alpha-napth. & sulf. acid - nitrite absent
no change with zinc- pos. nitrate reduction test

Fig. 28.7 Nitrate test

5. ***Pyrazinamidase test*** The enzyme pyrazinamidase hydrolyses pyrazinamide to ammonia and pyrazinoic acid, which is detected by adding ferric ammonium sulphate. This test is positive with *M. tuberculosis* and negative in *M. bovis.*

Anti-tuberculosis drug sensitivity tests

As drug resistance is a significant problem in tuberculosis, it is desirable to test the sensitivity of isolates as an aid to treatment. Sensitivity tests for *M. tuberculosis* are carried out using the following methods:

Conventional methods for testing drug susceptibility

1. **Absolute concentration method**—a number of media containing serial concentrations of the drugs are inoculated and the minimum inhibitory concentrations calculated.
2. **Resistance ratio method**—two sets of media containing graded concentrations of the drugs are inoculated, one set with the test strain and the other with a standard strain of known sensitivity.
3. **Proportion method**—the average sensitivity of the strain is indicated, taking into account the fact that any population will contain cells with varying degrees of sensitivity to a drug.

The disadvantage of these methods is the time taken for growth to occur before the reading can be taken.

Automated systems

One of these methods is the Gene Xpert MTB/RIF assay (Fig. 28.8), which is a fully automated molecular diagnostic test for tuberculosis. This is used more commonly now because rifampicin resistance can be detected simultaneously and because of the shorter turnaround time.

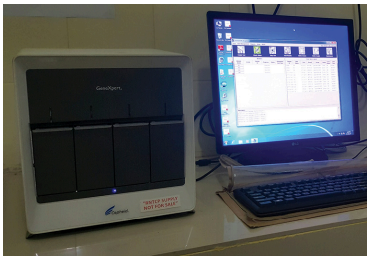


Fig. 28.8 Gene Xpert: rapid growth and resistance to rifampicin detected in a few days (*Source:* Intermediate Reference Laboratory for TB, SCB Medical College, Cuttack, Odisha)

Molecular methods (to detect MTB and drug resistance genes)

Nucleic acid amplification methods by polymerase chain reaction **(PCR)** and ligase chain reaction **(LCR)** are replacing culture methods, especially for extrapulmonary tuberculosis (e.g., cartridge-based nucleic acid amplification—**CB NAAT**) because they are rapid and can detect drug resistance genes simultaneously. An indigenous molecular detection method that was developed in India is Truenat (and Truenat MTB plus for the detection of drug resistance). It is similar in principle to CB NAAT and has now been approved by the WHO for use as a point-of-care tool for the detection of tuberculosis.

- **Transcription-mediated amplification (TMA)** , which targets ribosomal RNA, has been introduced as an improvement of PCR-based DNA amplification.
- **RFLP and IS fingerprinting** for epidemiological typing of strains have been referred to in the next section. Demonstration of mutations in specific drug sensitivity genes is a useful indicator of drug resistance. Such tests for rifampicin resistance are already available in diagnostic laboratories.
- **Line probe assay (LPA)** is used both for identification of MTC and detection of mutations associated with drug resistance genes.

Typing methods

They are used to geographically define the routes of transmission and dissemination in the environment and the source of infection in humans and

animals.

Molecular typing Most current typing methods are based on DNA fingerprinting, which is a powerful epidemiological tool for differentiating between strains of tubercle bacilli.

IS6110 Restriction Fragment Length Polymorphism (RFLP) typing The IS6110 is a target sequence in several methods currently used for molecular typing of *M. tuberculosis*. The patterns are strain-specific and can be used for fingerprinting (IS6110-based PCR fingerprinting).

Spoligotyping (spacer oligotyping) This is based on polymorphism in the direct repeat (DR) locus. This is the region present in all MTBC in a unique locus which contains well-conserved sequences. This is more useful in strains that have no or very few copies of IS6110. A great advantage of this method is its ability as a typing tool in non-viable cultures, AFB slides and paraffin-embedded tissues.

Insertion-sequence-based typing of non-tuberculous mycobacteria (IS-based typing of NTM) This method is based on differences in the insertion sequences of strains of avium complex. It can also be useful in typing *M. ulcerans*, *M. gordonae*, etc.

Immunodiagnosis

Serological tests are not useful in the diagnosis of tuberculosis, especially in endemic areas, and are not recommended.

Tuberculin test (also known as Mantoux test)

Demonstration of hypersensitivity to tuberculoprotein by **tuberculin testing** is a standard procedure to detect exposure to the bacilli.

In this test, 0.1 mL of PPD containing 5 TU (tuberculosis unit) is injected intradermally. After 48–72 hours, an induration of 10 mm or more at its widest point is taken as positive. Induration of 5 mm or less is considered negative and 6–9 mm equivocal. A PPD dose of 1 TU is used when extreme hypersensitivity is suspected. An increased dose of 10 or 100 TU is used when the 5 TU test is negative.

Interpretation

- **Positive** A positive tuberculin test indicates hypersensitivity to tuberculoprotein, denoting infection or prior immunisation with BCG. The test becomes positive 4–6 weeks after infection or immunisation and wanes gradually and disappears after 4–5 years. In endemic areas, the allergy is maintained by repeated contacts with the bacilli.
- **Negative** Persons who have not had contact or been exposed to the tubercle bacilli test negative. False negative tests (**anergy**) may be seen in miliary tuberculosis, convalescence from some viral infections like measles, lymphoreticular malignancy, sarcoidosis, severe malnutrition, immunosuppressive therapy or impaired cell-mediated immunity.

Uses Tuberculin testing may be used as an aid in diagnosing active infection in infants and young children. It also helps to determine the prevalence of infection in an area, or as an indication of successful vaccination. Tuberculin testing of cattle has helped in the control of bovine tuberculosis.

Gamma interferon assays

Interferon-gamma release assays (IGRAs) are in vitro blood tests of cell-mediated immune response; they measure T cell release of interferon-gamma following stimulation by antigens unique to *Mycobacterium tuberculosis* and a few other mycobacteria. In most individuals infected with *M. tuberculosis*, mononuclear cells in the blood release interferon-gamma when stimulated with antigens derived from *M. tuberculosis*. This test is useful to diagnose latent tuberculosis.

Animal inoculation
This method was used in the past to diagnose tuberculosis. However, with the advent of in vitro cultivation methods, it is no longer done, except for experimental purposes. The concentrated material is inoculated intramuscularly into the thighs of two healthy guinea pigs that are about 12 weeks old. Progressive loss of weight is an indication of infection. Infected animals show a positive tuberculin skin reaction.

Diagnosis of Extrapulmonary Tuberculosis

The general procedure is as for pulmonary tuberculosis. The **specimen** depends on the site of infection; urine, CSF, joint fluid, biopsy material,

blood or any other body fluid may be collected as a sample. **Microscopy** and **culture** are used for the diagnosis of extrapulmonary tuberculosis, though it is difficult to obtain conclusive results. This is because as the bacilli are present in fewer numbers in these lesions than in pulmonary disease. Molecular techniques can add to the diagnosis of extrapulmonary tuberculosis, done by conventional smear and culture, but cannot replace it.

- CSF from tuberculous meningitis often develops a spiderweb clot on standing, the examination of which may be more successful than that of the fluid. The use of PCR and DNA probes may be more efficient in detecting the bacilli.
- Bone marrow and liver biopsy specimens from miliary tuberculosis and blood from those with HIV co-infection are useful for culture. Pus from tuberculous abscess often yields positive results in smear and culture.
- Pleural effusion and other exudates may be collected with citrate to prevent coagulation. They may be directly cultured after centrifugation. Urinary excretion of bacilli in renal tuberculosis is intermittent. Hence it is advisable to test at least 2–3 first, whole-voided morning samples for culture. Each sample is centrifuged at 3,000 rpm for 30 minutes and the sediment used for culture after concentration.

Immunoprophylaxis

The **BCG (Bacille Calmette–Guerin) vaccine**, administered by intradermal injection of the live attenuated vaccine, was developed by Calmette and Guerin (1921). This is a strain of *M. bovis* attenuated by 239 serial subcultures in a glycerine–bile–potato medium over a period of 13 years. The immunity may last for 10–15 years and is similar to the immunity following natural infection.

Safety measures

The Lübeck disaster, in which 171 of the 250 children who were given oral immunisation developed clinical tuberculosis, faced severe criticism. This was later found to be due to live, virulent tubercle bacilli contaminating the

BCG vaccine.

Stringent safety measures have been enforced in the manufacture of the BCG vaccine.

Complications of BCG vaccine

The recognised complications of this vaccine are as follows:

- **Local** Abscess, indolent ulcer, keloid, tuberculides, confluent lesions, lupoid lesions and lupus vulgaris
- **Regional** Enlargement and suppuration of draining lymph nodes
- **General** Fever, mediastinal adenitis, erythema nodosum, tendency to keloid formation, and, very rarely, non-fatal meningitis; very few cases of progressive tuberculosis have been reported and are believed to have been in immunodeficient subjects
- **Efficacy** BCG may not offer protection from the risk of tuberculosis infection but gives protection to infants and young children against the more serious types of the disease, such as meningitis and disseminated tuberculosis

In endemic countries such as India, the BCG vaccine is administered to neonates by intradermal injection on the deltoid, immediately after birth or as early as possible, before the age of 12 months.

Contraindication to BCG

BCG is contraindicated for infants and children with active HIV disease (may be given with benefit to asymptomatic HIV-positive cases) and babies born to mothers with AFB-positive sputum. However, in the latter case (babies born to mothers with AFB-positive sputum), it may be given after a course of preventive chemotherapy.

Added advantages of BCG vaccine

BCG induces non-specific stimulation of the immune system, providing some protection against leprosy and leukemia. Multiple injections of BCG have been tried as adjunctive therapy in some malignancies. Some workers have reported that BCG is superior to PPD for tuberculin testing.

Chemoprophylaxis or Preventive Chemotherapy

Anti-TB drugs (usually only isoniazid) are administered to the following persons:

- Those with latent tuberculosis (asymptomatic, tuberculin-positive)
- Those at high risk of developing active tuberculosis
- Uninfected persons who are exposed to high risk of infection

- Infants of mothers with active tuberculosis
- Children living in the same house as a person with active tuberculosis
- HIV-infected contacts of persons with active tuberculosis

The drug of choice is **isoniazid 5 mg/kg daily for 6–12 months**. Trials have shown that this reduces the risk of developing active disease by 90 per cent.

Treatment

Chemotherapy has revolutionised the management of tuberculosis. The concept of sanatorium regimens, bed rest, fresh air and rich food, and operative interventions like artificial pneumothorax and thoracoplasty are no longer essential in adequately treated individuals.

Anti-tuberculosis drugs are of two types:

1. **Bactericidal** Of these, rifampicin (R) and pyrazinamide (Z) are called **sterilising drugs** because they effectively kill the bacilli in the lesions, whereas isoniazid is effective only against replicating bacilli and streptomycin (S) only against extracellular bacilli unable to sterilise the lesions on their own.
2. **Bacteriostatic** Ethambutol (E), along with bactericidal drugs, constitutes the first-line drug in anti-tuberculosis therapy. A short-course regimen of 6–7 months is effective and convenient. A combination of four drugs (HRZE) given three times a week during an initial intensive phase of two months, followed by 4–5 months of continuing phase with only two drugs (HR) three times a week has been found to be effective. RNTCP follows the **Directly Observed Treatment, Short course (DOTS).**

Treatment of pediatric tuberculosis

All forms of TB except TB meningitis, bone and joint TB (osteoarticular TB) are treated with rifampicin, isoniazid, pyrazinamide and ethambutol in the intensive phase followed by continuation phase of four months of rifampicin and isoniazid. In TB meningitis, bone and joint TB (osteoarticular TB), the continuous phase is for ten months with rifampicin and isoniazid.

In case of drug-resistant TB, the guidelines discussed next are followed under intense monitoring and supervision.

Drug Resistance

Drug-resistant tuberculosis has become a problem in high-TB-burden countries, including India. This is due to **mutations, with an approximate rate of 1 in 10^8 cell divisions**. Multiple factors have led to the emergence of MDR-TB (multidrug-resistant tuberculosis). Lapses in prescribing practices, drug delivery and patient compliance have led to the build-up of resistance in the bacilli over the years, reducing the efficacy of treatment.

In addition to the classification of drug resistance as primary and acquired resistance, the World Health Organization classifies drug resistance in tuberculosis as follows:

1. ***Resistance in newly treated*** This occurs when the patient is infected with a strain of the tubercle bacilli that is already resistant.
2. ***Resistance in previously treated (secondary, post-treatment)*** This occurs when the infecting strain, which was initially sensitive, becomes resistant. This usually is a result of improper or inadequate treatment. This is the more common type of resistance.

When acquired, resistant strains become increasingly common in an area and the chance of new patients presenting with primary resistance increases. When an infecting strain acquires resistance to one drug, the chance of it becoming resistant to other drugs increases, unless the treatment schedule contains an adequate number of effective drugs.

Multidrug-resistant tuberculosis (MDR-TB)

The consequence of unchecked drug resistance has resulted in the emergence and spread of **multidrug-resistant tuberculosis (MDR-TB).** In tuberculosis, it specifically refers to **resistance to rifampicin and isoniazid**, with or without resistance to one or more of the other drugs. **This is because R and H form the sheet anchor of short-term chemotherapy and any strain resistant to both these drugs is unlikely to respond to treatment.**

MDR-TB is a global problem, menacing the developed and undeveloped nations alike. It may be primary or acquired. Its presence in those with concomitant HIV infection makes it more dangerous. When first-line drugs become ineffective, second-line drugs must be tried. Large numbers of old

and new drugs are being used: quinolones, aminoglycosides, macrolides, para-aminosalicylic acid, thiacetazone, cycloserine, capreomycin and others. They are unsatisfactory, being much less effective, costlier, more toxic and requiring prolonged treatment schedules.

Extensively drug-resistant tuberculosis (**XDR-TB)** is resistant to first-line (MDR) plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin or capreomycin).

REVISED NATIONAL TUBERCULOSIS CONTROL PROGRAMME (RNTCP)

RNTCP was implemented in India in 1992. The aim was to detect new cases through **designated microscopy centres** (DMC), and provide standardised treatment and proper diagnostic facilities. This was based on the Directly Observed Treatment, Short Course (DOTS) strategy of the WHO. The treatment is started following diagnosis made primarily by morning and spot sputum microscopy. This is made available free of cost to patients at DMCs. Treatment is provided under direct observation by a DOT provider at the DOTS centre near the patients' home. This strategy can prevent the emergence of drug resistance by ensuring the patient's compliance. RNTCP was renamed as **National TB Elimination Programme (NTEP)** from 1 January 2020 by the Govt. of India. The revised programme encompasses strategies to promote quality of life and health by preventing, controlling and finally, eliminating TB.

India DOTS is the fastest expanding programme in the world. For the purpose of treatment, based on the sputum smear positivity and the seriousness of the disease, regimens are given as category I, II and III of treatment. The algorithms for laboratory diagnosis and treatment strategies are standardised. It also identifies accredited laboratories for drug susceptibility testing.

DOTS relies on treatment with the first-line drugs rifampicin and INH (isoniazid).

DOTS-Plus refers to DOTS programmes that add components for MDR-TB diagnosis and treatment using quality-assured culture and drug susceptibility testing (DST). Proper triage of patients for culture and DST testing and management under DOTS-Plus is done in coordination with

National and Supra-National Reference Laboratories.

RNTCP—Standardised Treatment Regimen (Cat IV)

This regimen is for the treatment of MDR-TB cases (and those with rifampicin resistance) under the RNTCP programme (Table 28.4). Cat IV regimen comprises 6 drugs—kanamycin, ofloxacin (levofloxacin), ethionamide, pyrazinamide, ethambutol and cycloserine—during the 6–9 months of the intensive phase, and 4 drugs—ofloxacin (levofloxacin), ethionamide, ethambutol and cycloserine—during the 18 months of the continuation phase. *p*-aminosalicylic acid (PAS) is included in the regimen as a substitute drug if any bactericidal drug (K, Ofl, Z and Eto) or 2 bacteriostatic (E and Cs) drugs are not tolerated.

Table 28.4 Alternative method of grouping anti-TB agents

Group 1: First-line oral anti-TB agents	Isoniazid (H); rifampicin (R); ethambutol (E); pyrazinamide (Z)
Group 2: Injectable anti-TB agent	Streptomycin (S); kanamycin (Km); amikacin (Am); capreomycin (Cm); viomycin (Vm)
Group 3: Fluoroquinolones	Ciprofloxacin (Cfx); ofloxacin (Ofx); levofloxacin (Lvx); moxifloxacin (Mfx); gatifloxacin (Gfx)
Group 4: Oral second-line anti-TB agents	Ethionamide (Eto); prothionamide (Pto); cycloserine (Cs); terizadone (Trd); *para*-aminosalicylic acid (PAS)
Group 5: Agents with unclear efficacy (not recommended by WHO for routine use in MDR-TB patients)	Clofazimine (Cfz); linezolid (Lzd); amoxicillin/clavulanate (Amx/Clv); thioacetazone (Thz); imipenem/cilastatin (Ipm/Cln); high-dose Isoniazid (high-dose H); clarithromycin (Clr)

Source: Revised National Tuberculosis Control Programme DOTS-Plus Guidelines

The Stop TB Strategy of the World Health Organization

To reduce the global burden of TB **and for a TB-free world**, in line with the Millennium Development Goals and the Stop TB Partnership targets, the WHO has laid out the following objectives:

- Achieve universal access to high-quality care for all people with TB.
- Reduce the human suffering and socioeconomic burden associated with TB.
- Protect vulnerable populations from TB, TB/HIV and multidrug-resistant TB.

- Support development of new tools and enable their timely and effective use.
- Protect and promote human rights in TB prevention, care and control.

National Strategic Plan, India (2017–2025)

The Govt. of India plans to make India TB free by 2025. The strategy is as follows:
- Including private health care settings
- Tracing the people with TB who have been missed by the care provided through DOTS
- Active case finding among key populations
- Preventing the development of active TB in people with latent TB in high-risk groups like HIV

Key points

- *Mycobacterium tuberculosis* is an obligatory aerobic, non-motile, non-sporing, rod-shaped bacterium whose cell wall contains an abundance of lipids (mycolic acids). It retains strong carbol fuchsin dye during decolourisation with acid and alcohol in the Ziehl–Neelsen (ZN) staining technique (*Mycobacterium tuberculosis* is acid and alcohol fast by this staining technique). It grows very slowly, taking several weeks to form a visible colony on enriched culture media.
- *Mycobacterium tuberculosis* causes tuberculosis (TB) in humans; this is the leading cause of bacteria-related deaths worldwide.
- TB is transmitted by aerosols from an infected individual. Inhaled bacteria penetrate the alveoli and are ingested by alveolar macrophages. Bacteria then grow intracellularly and slowly.
- Cell-mediated immunity is the primary immune response that destroys the organism inside macrophages. Individual susceptibilities to TB reflect differences in the efficacy of an individual's cell-mediated response to infection.
- TB affects primarily the lungs. It can disseminate to the CNS, bone, gut and other organs of the body.
- It is diagnosed by the following methods:
    - Sputum smear examination by staining with ZN method; acid- and alcohol-fast bacilli appear as long, thin and pink (sometimes beaded) rods.
    - Culture of sputum on Löwenstein–Jensen or Middlebrook medium; this may take up to 6–8 weeks to yield positive results.
    - Automated systems have improved the turnaround time of culture and sensitivity.
    - The tuberculin skin test is a sign of exposure to the organism.
    - PCR can be used to detect *Mycobacterium tuberculosis* DNA in sputum and other specimens.
- Individuals suspected to have the disease should be treated with multiple antibiotics. MDR and XDR strains are a cause of concern in treatment.

- DOTS under RNTCP in India ensures that patients receive proper therapy.

Questions

Essays

1. Classify mycobacteria. Describe the laboratory diagnosis of pulmonary tuberculosis.
2. Enumerate the infections caused by *M. tuberculosis* and describe the laboratory diagnosis of extrapulmonary tuberculosis.

Short Answers

1. Principle of acid-fast staining
2. Kinyoun's method of acid-fast staining
3. BCG vaccine
4. Tuberculin test
5. Methods of anti-tubercular susceptibility tests
6. MDR and XDR-TB
7. DOTS
8. Automated methods for culture of mycobacteria

Part III: Bacteriology

Chapter 29

Mycobacteria II

Chapter navigation

- Atypical mycobacteria
  - Classification
  - Infections
- Leprosy
  - Epidemiology
  - Treatment
  - Elimination programme

INTRODUCTION

Mycobacteria other than tubercle bacilli occasionally cause human disease resembling tuberculosis, especially in immunocompromised individuals and in post-organ-transplant and HIV patients. These organisms are called **non-tuberculous mycobacteria (NTM)** earlier also called 'atypical', 'anonymous' or 'MOTT'—mycobacteria other than tubercle bacilli. Over 80 species of NTM are found worldwide in the soil and in animals.

Saprophytic mycobacteria such as *M. phlei* are incapable of infecting human beings or animals and are distinct from NTM.

Runyon Classification

NTM have been classified into four groups by Runyon (1959) based on pigment production and the rate of growth: Group I photochromogens, Group II scotochromogens, Group III non-photochromogens and Group IV rapid growers (Table 29.1).

Table 29.1 Runyon classification

Group	Nomenclature	Growth characteristics	Members of clinical importance	Diseases
I	Photochromogens	No pigment in the dark, become pigmented on exposure to light	*M. kansasii*, *M. marinum*	Pulmonary disease resembling tuberculosis; swimming pool granuloma
II	Scotochromogens	Pigmented in the dark	*M. scrofulaceum*, *M. gordonae*	Cervical lymphadenitis; mostly contaminant from tap water
III	Non-photochromogens	No pigment even on exposure to light	*M. avium*, *M. intracellulare*, *M. ulcerans*, *M. xenopi*	Disseminated disease in AIDS patients; Buruli ulcer; chronic lung disease
IV	Rapid growers	Growth within seven days	*M. fortuitum*, *M. chelonae*, *M. smegmatis*, *M. phlei*	Chronic abscess; saprophytes

Group I—photochromogens

These strains form colonies that do not produce pigment in the dark but when the young culture is exposed to light for one hour in the presence of air, and re-incubated for 24–28 hours, a yellow-orange pigment appears. The important species in this group are *M. kansasii, M. marinum* and *M. simiae.* They can cause human infections.

- ***M. kansasii*** causes chronic pulmonary disease resembling tuberculosis, usually affecting the upper lobes, with cavity formation and scarring. ***M. marinum,*** which causes a warty skin lesion (**swimming pool** or **fish tank granuloma**), closely resembles *M. kansasii* but can be differentiated by its poor growth at 37°C and by biochemical tests.
- Others include ***M. simiae*** and niacin-negative ***M. asiaticum***. They have subsequently been associated with pulmonary disease in human beings.

Group II—scotochromogens

These strains form pigmented colonies (yellow-orange-red) even in the dark. They are widely distributed in the environment and sometimes contaminate cultures of tubercle bacilli.

- ***M. scrofulaceum*** may cause scrofula (cervical adenitis) in children.
- ***M. gordonae***, often found in tap water (hence called '**the tap water scotochromogen**'), is a common contaminant in clinical

specimens and a rare cause of pulmonary disease.

- ***M. szulgai*** is scotochromogenic when grown at 37°C and photochromogenic at 25°C.

Group III—non-photochromogens

These strains do not form pigments even on exposure to light. Colonies may resemble those of the tubercle bacilli. The medically important species are *M. avium, M. intracellulare, M. xenopi* and the skin pathogen, *M. ulcerans*.

- ***M. avium*** is one of the most common opportunistic human pathogens. It causes natural tuberculosis in birds and lymphadenopathy in pigs.
- ***M. intracellulare*** and *M. avium* are similar and are hence considered as one group, the ***M. avium*** **complex (MAC).** They cause lymphadenopathy, pulmonary lesions or disseminated disease, particularly in AIDS patients. *M. intracellulare* is commonly known as the **Battey bacillus** because it was first identified as a human pathogen at the Battey State Hospital for Tuberculosis, Georgia, USA.
- ***M. xenopi***, originally isolated from toads, may occasionally cause chronic lung disease in human beings. *M. xenopi* and *M. avium* are thermophiles, capable of growth at 45°C.

Group IV—rapid growers

This is a heterogeneous group of mycobacteria capable of rapid growth, colonies appearing within seven days of incubation at 37°C or 25°C (Fig. 29.1).

Fig. 29.1 Growth on LJ medium (*M. fortuitum*)

- Within the rapid growers, there exist photochromogenic, scotochromogenic and non-chromogenic species. Chromogenic rapid growers are mostly saprophytes (e.g., *M. phlei*). The medically important species are ***M. fortuitum*** and ***M. chelonae***, both of which can cause **chronic abscesses** in human beings. Outbreaks of abscesses **following injection of vaccines** and other preparations contaminated by these mycobacteria have been reported on a number of occasions. The bacilli are found in the soil, and infection usually follows some injury. No effective chemotherapy is available.
- ***M. smegmatis***, commonly considered as a saprophyte in smegma, is seldom seen in that location. It is a frequent isolate from soft tissue lesions that follow trauma or surgery.
- Some non-cultivable or poorly growing mycobacteria identified from the blood of AIDS patients have been characterised by their 16S RNA base sequences.

> A rapid grower, ***M. vaccae*** is reported to be an immunomodulator capable of inhibiting tissue-destroying hypersensitivity responses and stimulating protective immune processes in tuberculosis. Clinical trials of the *M. vaccae* vaccine as an adjuvant to chemotherapy in tuberculosis are underway.

MYCOBACTERIAL SKIN INFECTIONS

Cutaneous infections occur in leprosy or tuberculosis, either as localised disease or as part of a generalised infection.

Exclusively, skin pathogens include *M. ulcerans* (Group III of Runyon) and *M. marinum* (Group I of Runyon). They cause chronic ulcers and granulomatous lesions on the skin (Table 29.2). **Systemic invasion does not occur and the regional lymph glands are not involved**. Cutaneous localisation is because they multiply optimally at skin temperature. Infection with *M. marinum*, but not with *M. ulcerans*, may cause a low-grade tuberculin reaction.

Table 29.2 Differentiation of characteristics of *M. ulcerans* and *M. marinum*

Characteristic	*M. ulcerans*	*M. marinum*
Distribution	Tropics	Temperate zone
Clinical course	Chronic progressive ulcer	Self-limited ulcer

Bacilli in ulcer	Abundant	Scanty
Rate of growth	Slower, 4–8 weeks	Faster, 1–2 weeks
Growth at 25°C	–	+
Growth at 37°C	–	+
Culture film	Bacilli in cords	No cord formation
Pigment in light	–	+
Mouse footpad lesion	Edema, rarely ulcer	Marked inflammation—purulent ulcer

***M. ulcerans* (Buruli ulcer)** This is commonly seen in Uganda, Congo, Nigeria, Mexico, Malaysia and New Guinea. Ulcers are usually seen on the legs or arms following minor injuries. After an incubation period of a few weeks, indurated nodules appear, which break down to indolent ulcers which slowly extend under the skin.

Initially, smears from the edge of the ulcer show large clumps of bacilli which are acid-fast and alcohol-fast. Later, the immunoreactive phase sets in and the bacilli disappear. The ulcers heal with disfiguring scars.

A toxin produced by *M. ulcerans* causes inflammation and necrosis.

***M. marinum* (swimming pool granuloma)** This is a natural pathogen of cold-blooded animals and causes tuberculosis in fish and amphibians. Human infection originates from contaminated swimming pools or fish tanks. The lesion forms an indolent ulcer, following abrasions on elbows, knees, ankles, nose, fingers or toes. Bacilli are scanty in smears from ulcers.

Epidemiology

An inverse relationship seems to exist between tuberculosis and the disease caused by NTM. Where tuberculosis is endemic, opportunistic mycobacterial disease is rare.

Laboratory Diagnosis

Some opportunistic species may cause infection and occasionally, contaminate the cultures. They may be found in tap water (for example, *M. avium, M. kansasii, M. xenopi*). These may cause problems in the laboratory where they may be mistaken for the tubercle bacilli in acid-fast smears, and false positive reports are given. The two can be differentiated by growth

characters and biochemical tests (Table 29.3).

Table 29.3 Differentiation between tubercle bacilli and some species of atypical mycobacteria

Test	*M. tuber-culosis*	*M. bovis*	*M. microti*	*M. kansasii*	*M. marinum*	*M. scrofu-laceum*	*M. avium-intracellulare* complex	*M. fortuitum*	*M. chelonei*	*M. phlei*	*M. smegmatis*
Growth in 7 days	–	–	–	–	–	–	–	+	+	+	+
Growth at 25°C	–	–	–	+	+	+	±	+	+	+	+
Growth at 37°C	+	+	+	+	±	+	+	+	+	+	+
Growth at 45°C	–	–	–	–	–	–	±	–	–	+	+
Pigment in dark	–	–	–	–	–	+	–	–	–	+	–
Pigment in light	–	–	–	+	+	+	–	–	–	+	–
Growth in the presence of *p*-nitrobenzoic acid 500 mg/mL (PNB)	–	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	–	+	+	+	+
Niacin	+	–	±	–	–	–	–	–	–	–	–
Nitrate reduction	+	–	–	+	–	–	–	+	–	+	+

Treatment

Most environmental mycobacteria are resistant to the usual anti-tuberculous drugs, though pulmonary disease caused by *M. avium* complex or *M. kansasii* may respond to prolonged treatment with rifampicin, isoniazid and ethambutol. Various combinations of drugs including rifabutin, clofazimine, quinolones and newer macrolides may be used.

MYCOBACTERIUM LEPRAE

Leprosy is a disease of antiquity, having been recognised since the Vedic times in India and the biblical times in the Middle East. The lepra bacillus was first observed by **Hansen in 1868**. It is predominantly a disease of the developing world.

Morphology

M. leprae is a straight or slightly curved rod, 1–8 × 0.2–0.5 μm in size, showing considerable morphological variation. Polar bodies and other intracellular elements may be present. Clubbed forms, lateral buds or branching may be observed.

It is acid-fast but less so than the tubercle bacillus. Hence 5% sulphuric acid instead of 20% is used for decolourisation after staining with carbol fuchsin. The bacilli are seen singly and in groups, intracellularly or lying free outside the cells. They are bound together by a lipid-like substance, the glia. These masses are known as '**globi**'. The parallel rows of bacilli in the

globi present a '**cigar bundle**' appearance. In tissue sections, the clumps of bacilli resemble cigarette ends. The globi appear in undifferentiated histiocytes called Virchow's **lepra cells** or **foamy cells** (Fig. 29.2).

It has not been possible to cultivate lepra bacilli either in bacteriological media or in tissue culture.

Generation time Lepra bacilli have a generation time of 12–13 days on an average (the range being 8 to 42 days), which is extremely long in comparison to *Mycobacterium tuberculosis* that doubles in 14 hours and *E. coli* which has a generation time of just 20 minutes.

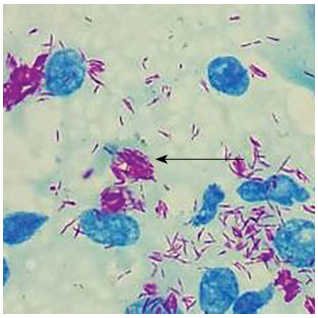


Fig. 29.2 *M. leprae* acid-fast pink bacilli with typical 'globi' arrangement (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Methods of Cultivation in Animals

i) **Footpad of mice** Shepard (1960) discovered that lepra bacilli could multiply in the footpads of mice at a low temperature (20°C). This has become the **standard procedure for experimental work with the bacillus**. Granuloma develops at the site of inoculation in 1–6 months. In thymectomised mice or following the administration of an antilymphocyte serum (to suppress CMI), generalised infection is produced, simulating lepromatous leprosy.

ii) **Nine-banded armadillo** The *Dasypus novemcinctus* is highly susceptible to infection with the lepra bacilli. Generalised infection occurs with extensive multiplication of the bacilli and the production of lesions typical of lepromatous leprosy. 'Natural disease' has also been identified in chimpanzees and mangabey monkeys from West Africa but it is not known whether they have any relevance to human infection.

Resistance

Lepra bacilli have been found to remain viable in a warm, humid environment for 9–16 days and in moist soil for 46 days. They survive exposure to direct sunlight for two hours and ultraviolet light for 30 minutes.

The whole genome of *M. leprae* has been mapped and the genes for its major protein antigens have been cloned and sequenced.

LEPROSY

Leprosy is a chronic granulomatous disease of humans, primarily involving the skin, peripheral nerves and nasal mucosa but capable of affecting any tissue or organ.

According to the WHO, leprosy is classified on the basis of clinical manifestations and skin smear results. In the classification based on skin smears, patients showing negative smears at all sites are grouped as **paucibacillary leprosy (PB) (tuberculoid leprosy)** while those showing positive smears at any site are grouped as having **multibacillary leprosy (MB) (lepromatous leprosy).**

Lepromatous leprosy, also known as '**multibacillary disease**', is seen when host resistance is low. The bacilli are seen in large numbers or as globi inside lepra cells or extracellularly (Fig. 29.3). Superficial nodular lesions (lepromata) develop, which consist of granulation tissue containing a dense collection of vacuolated cells in different stages of development from mononuclear cells to lepra cells. Bacilli are shed in large numbers in nasal and oral secretions. The reticuloendothelial system, eyes, testes, kidneys and bones are also involved. Erosion into bones and cartilage of the nose gives the person a lion-like appearance (**leonine facies)**. The lepromatous type is more infective than the other types. Cell-mediated immunity is deficient and the lepromin test is negative in lepromatous leprosy. There is an exaggerated and broad humoral immune response. **Autoantibodies are common.** Most cases show biological false-positive reaction in standard serological tests for syphilis.

Fig. 29.3 Lepromatous leprosy with leonine facies
(*Source:* Public Health Image Library, ID 15502 / Dr. Andre J. Lebrun / CDC)

The following complications may occur due to untreated lepromatous leprosy:

- Foot drop
- Plantar ulcerations
- Loss of distal digits
- Loss of nasal cartilage
- Lagophthalmus and corneal insensitivity (due to cranial nerve involvement)
- Mild to severe testicular dysfunction in males, leading to infertility
- Amyloidosis
- Nerve abscesses

Tuberculoid leprosy is seen in patients with a high degree of resistance. The skin lesions are few and sharply demarcated, consisting of macular anaesthetic patches (Fig. 29.4). Neural involvement occurs early and may be pronounced, leading to deformities, particularly in the hands and feet. Bacilli are scanty in the lesions and infectivity is minimal. This is known as **paucibacillary disease**. Cell-mediated immunity is adequate and the lepromin test is positive. Anti-mycobacterial and autoimmune antibodies are rare, hence the prognosis is good.

Fig. 29.4 Tuberculoid leprosy: sharply demarcated, macular patches (*Source:* Public Health Image Library, ID 16518 / Dr. Andre J. Lebrun / CDC)

Borderline or **dimorphous** type refers to lesions possessing characteristics of both tuberculoid and lepromatous types. It may shift to the lepromatous or tuberculoid part of the spectrum depending on chemotherapy or alterations in host resistance. Some of the differences are shown in Table 29.4.

Table 29.4 Characterisation of the different types of leprosy

	Tuberculoid	Borderline	Lepromatous
Lepra bacilli in tissues	±	+	+++
Lepromin test	++	±	–
Mycobacterial antibodies	±	+	+++
Lymphocytic infiltration of lesions	+++	+	–
Plasma cells in lymphoid tissue	+	+	+++

The **indeterminate** type is the early unstable tissue reaction which is not characteristic of either the lepromatous or the tuberculoid type. In several persons, the indeterminate lesions undergo spontaneous healing. In others, the lesions may progress to the tuberculoid or lepromatous types.

Epidemiology

Leprosy is an exclusively human disease restricted to the developing world, Asia, Africa, Latin America and the Pacific. In India, leprosy is present in all states and territories. Orissa and Bihar have the highest prevalence (> 5 per 1,000 population) and Haryana the least (< 0.1 per 1,000).

The exact mode of transmission is not clear. Very large numbers of bacilli are shed in nasal secretions (up to 8×10^8 bacilli in one nose-blowing). The

mode of entry may be through the respiratory tract or through the skin. Asymptomatic infection appears to be quite common in endemic areas and may have a role in transmission. Leprosy is not highly communicable. The disease develops in only about 5 per cent of spouses living with leprosy patients.

The incubation period is very long and averages 2–5 years. It is generally held that intimate and prolonged contact is necessary for infection to take place and is more likely if contact occurs during childhood.

Immunity

A high degree of **innate immunity** against lepra bacilli seems to exist in human beings and so only a minority of those infected develop clinical disease. Infection with the lepra bacilli induces both humoral and cellular immune response. Antibodies do not have any effect on the bacilli, while cellular immune mechanisms are capable of destroying them. **The type of leprosy in an individual is determined by the status of cell-mediated immunity in that person**.

When **cell-mediated immunity** is deficient, the lepromatous type of disease develops. Delayed hypersensitivity to the lepra bacillus protein is absent.

The deficiency in cell-mediated immunity is specific to the lepra bacillus antigens. Lepromatous patients have large numbers of $CD8^+$ (suppressor) lymphocytes in circulation.

The lymphocytes present in skin granulomas are almost exclusively $CD8^+$ cells, in contrast to tuberculoid lesions, which contain predominantly $CD4^+$ cells. In lepromatous leprosy, there is **extensive polyclonal B cell activation**. Large amounts of non-protective antibodies are produced which include both **anti-mycobacterial antibodies** and **autoimmune antibodies.** The albumin: globulin ratio is reversed.

Genetic predisposition There is evidence of genetic effect in the pattern of response to the lepra bacillus infection**. HLA-DR2 is seen preponderantly in persons with the tuberculoid type** of reaction, while **HLA-MTI and HLA-DQ1 are associated with lepromatous disease.**

Lepra Reactions

Though leprosy is a chronic disease, its course may be interspersed with acute exacerbations which are of an allergic nature. Two types of such reactions occur.

- **Type 1**, the **reversal reaction** or the **lepra reaction,** is seen mostly in borderline leprosy. There is erythema and swelling, along with pain and tenderness. A similar clinical picture is seen in the **downgrading reaction** which occurs usually in untreated or pregnant patients. Here, the biopsy of the lesions shows a shift to the lepromatous pattern, reflecting a decrease in CMI.
- **Type 2** reaction or **erythema nodosum leprosum** (ENL) occurs in the LL and BL types, usually a few months after institution of chemotherapy. Crops of tender, inflamed subcutaneous nodules appear, with fever, lymphadenopathy and arthralgia. This is an **Arthus type response to antigens released from dead lepra cells** and is characterised by neutrophil infiltration and IgG and complement deposition in the lesions.

Lepromin test

The lepromin test was first described by Mitsuda in 1919. **Standard lepromins are prepared from armadillo-derived lepra bacilli (lepromin-A).**

- **Early reaction** of **Fernandez** consists of erythema and induration developing in 24–48 hours and usually remaining for 3–5 days. This is analogous to the tuberculin reaction.
- **Late reaction** of **Mitsuda**, is the second and more specific test, starting in 1–2 weeks, reaching a peak in four weeks and gradually subsiding in the next few weeks. The reaction consists of an indurated skin nodule, which may ulcerate. Histologically, lymphocytes, epithelioid cells and giant cells are characteristically present. The Mitsuda late reaction does not indicate pre-existing DTH but is a measure of the CMI.

Applied importance of the lepromin test

The lepromin test is not used to diagnose leprosy, nor does it indicate prior contact with the lepra bacillus. Lepromin test is used to:

- **Classify the lesions** of leprosy patients. The lepromin test is positive in tuberculoid, negative in lepromatous and variable in dimorphous and indeterminate types of disease.
- **Assess the prognosis** and response to treatment. Conversion to lepromin-positive status during treatment is evidence of improvement.
- **Assess the resistance** of individuals to leprosy.
- **Identify candidate** lepra bacilli.

Laboratory Diagnosis

Diagnosis of leprosy is based on at least one of three cardinal signs: (i) hypopigmented skin patch with loss of or reduced sensation, (ii) enlarged nerve and (iii) slit-skin smear positive for leprosy bacilli (WHO recommendation). For routine examination, specimens are collected from the nasal mucosa, skin lesions and ear lobules.

1. Specimen

- **Nasal smear** A blunt, narrow scalpel introduced into the nose is used to remove a piece of mucous membrane, which is transferred to a slide and teased out into a uniform smear. Alternately, the patient is asked to blow into a tissue paper and the material is scraped by a scalpel and smeared on a slide.
- **Slit skin smear** The skin over the earlobe is pinched up tight to minimise bleeding and a cut, about 5 mm long made with a scalpel, deep enough to get into the infiltrated layers. After wiping off blood or lymph that may have exuded, the scalpel blade is turned transversely to scrape the sides and bottom of the cut so as to obtain a little tissue pulp which is smeared uniformly on a slide. About 5–6 different areas of the skin are sampled, including the skin over the buttocks, forehead, chin, cheek and ears.
- **Nerve biopsy** A sample is collected from thickened nerves and submitted for histopathological examination.

2. **Microscopy** The smears are stained by the **Ziehl–Neelsen technique** using 5% instead of 20% sulphuric acid for decolourisation. Biopsy of the nodular lesions, thickened nerves and lymph node puncture may be

necessary in some cases.

The smears are graded, based on the number of bacilli as follows:
1–10 bacilli in 100 fields = 1+
1–10 bacilli in 10 fields = 2+
1–10 bacilli per field = 3+
10–100 bacilli per field = 4+
100–1,000 bacilli per field = 5+
More than 1,000 bacilli, clumps = 6+
and globi in every field

The **bacteriological (bacterial) index (BI)** is calculated by totalling the number of pluses (+s) scored in all the smears and diving that number by the number of smears. For calculating BI, a minimum of four skin lesions, a nasal swab and both the ear lobes have to be examined.

The **morphological index (MI)** is expressed as the percentage of solid fragmented granular bacilli (SFGB) or uniformly stained bacilli out of the total number of bacilli counted.

3. **Serology It is** not specific, hence is not done routinely.

4. **Molecular methods** Molecular studies are still in the research stage, and have not been utilised as diagnostic tools.

Treatment

World Health Organization's **limited duration multidrug therapy** has led to the adoption of the following WHO classification for treatment purposes:

- Single lesion paucibacillary (SLPB)
- Paucibacillary (PB), i.e., those with two to five lesions
- Multibacillary (MB), i.e., those with six or more lesions

Dapsone was the first effective chemotherapeutic agent against leprosy. Its use as a monotherapy for several years led to the development of resistant strains of lepra bacilli. In view of this, **multiple drug therapy (MDT**) is now recommended in leprosy, as in tuberculosis. The current recommendation is as follows:

- **MB leprosy** The standard adult treatment regimen is:

Rifampicin: 600 mg once a month
Clofazimine: 300 mg once a month; 50 mg daily
Dapsone: 100 mg daily

(Ethionamide or prothionamide may be added to this regimen or substituted for clofazimine)
Duration: 12 months

- **PB leprosy** The standard adult treatment regimen is:

Rifampicin: 600 mg once a month
Dapsone: 100 mg daily
Duration: 6 months
A minimum follow-up of four years for paucibacillary and eight years for multibacillary cases is necessary to detect any relapse.

Prophylaxis

The role of chemoprophylaxis is debatable. Instead, case finding and adequate therapy have been the methods employed for prophylaxis.

Long-term chemoprophylaxis has given encouraging results in childhood contacts of newly diagnosed infectious cases in India and the Philippines.

BCG vaccine No specific vaccine is available. Since there is some degree of antigenic relationship between the lepra and tubercle bacilli, field trials with different leprosy vaccines (BCG + killed lepra bacilli; ICRC bacillus) were conducted, but without conclusive evidence.

Leprosy vaccine An indigenous leprosy vaccine, ***mycobacterium indicus pranii*****,** has been approved by the Drug Controller General of India (DGCI) and Food and Drug Administration (FDA) department of the US. It is still in its pilot phase, and if found successful, will be rolled out in high-prevalence states.

National Strategies to Control Leprosy in India

The National Leprosy Eradication Programme of the Ministry of Health and Family Welfare, Govt. of India, is the hub for all national programmes to detect, treat and control leprosy. The programme is implemented by Indian States/UTs. World Health Organization and the International Federation of Anti-leprosy Associations (ILEP) support the programme along with some non-governmental organisations.

There are central institutes that undertake research and programme

implementation in several states.

- Central Leprosy Teaching and Research Institute (CLTRI) at Chengalpattu, Tamil Nadu
- Japanese Leprosy Mission for Asia (JALMA) is the National (JALMA) Institute for Leprosy and Other Mycobacterial Diseases situated in Agra, Uttar Pradesh, which conducts research on leprosy, tuberculosis and HIV; it is currently one of the ICMR institutes
- Regional Leprosy Training and Research Institute, Aska, Odisha, which is the central institute that works as a referral centre for the management of difficult-to-diagnose leprosy cases, and provides physiotherapy

Elimination of leprosy

Leprosy Elimination Campaign has been introduced by Central Leprosy Division (CLD) of the Govt. of India's Ministry of Health and Family Welfare. It aims to bring about a reduction in annual new cases of Grade II disability to less than 1 case per million population at the national level.

Key points

- Non-tuberculous mycobacteria have been classified by Runyon's classification into:
  - Photochromogens: *Mycobacterium kansasii* and *Mycobacterium marinum*
  - Scotochromogens: *Mycobacterium scrofulaceum* and *Mycobacterium gordonae*
  - Non-chromogens: *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium xenopi* and *Mycobacterium ulcerans*
  - Rapid growers: *Mycobacterium fortuitum* and *Mycobacterium chelonae* (*M. chelonei*)
- Infections by the NTM include:
  - Lung infections, which manifest as tuberculosis-like disease (with possible dissemination in patients with AIDS)
  - Skin infections (Buruli ulcer, swimming pool granuloma, injection abscess), especially in already diseased tissue or immunocompromised individuals
  - Lymphadenopathy, especially of cervical nodes in children
- Characterisation of isolates by growth rate and pigment formation in light (photochromogen), dark (scotochromogen) or no pigment at all (non-photochromogen) is the mainstay of laboratory diagnosis.

- Infected individuals need to be treated with multiple anti-mycobacterial drugs (they frequently tend to be resistant to the first-line, anti-mycobacterial drugs). Azithromycin or other macrolides need to be given prophylactically to individuals infected with HIV.
- *Mycobacterium leprae* is an obligate, intracellular organism, which is acid-fast with 5% sulphuric acid and is non-alcohol fast.
- Aerosol droplets and direct contact with infected skin contribute to transmission.
- Leprosy manifests as a spectrum of diseases. The least severe form is tuberculoid tuberculoid (TT) and the most severe form is lepromatous lepromatous (LL). In between are the borderline forms (tuberculoid and lepromatous).
- The severity of leprosy is inversely related to the status of cell-mediated immunity. Delayed-type hypersensitivity response to *M. leprae* protein antigens (lepromin) is good in TT but absent in LL.
- Diagnosis can be established by clinical signs and microscopy. Acid-fast staining of tissue reveals typical long, thin bacilli or globule clusters. The organism grows in the footpads of mice (especially for antibiotic sensitivity testing) and the nine-banded armadillo, but not in cell-free or tissue culture systems.

Questions

Essays

1. Describe the clinical manifestation, laboratory diagnosis and epidemiology of lepromatous leprosy.
2. Discuss the immunological basis of tuberculoid and lepromatous leprosy. Describe the laboratory confirmation of tuberculoid leprosy.

Short Answers

1. Non-tuberculous mycobacteria and infections caused by them
2. Lepromin test
3. Cultural characteristics of non-tuberculous mycobacteria

Part III: Bacteriology

CHAPTER 30

Enteric Bacilli (Gram-Negative Rods)

Chapter navigation

- Enterobacteriaceae
- Classification
  - Tribes, genera and species
- Antigenic structure
- Diseases
- Pathogenesis
- Clinical features
- Diagnosis
- Treatment

INTRODUCTION

Enterobacteriaceae is a large group of non-sporing, non-acid-fast, gram-negative rods that are heterogeneous. They are found in the gut of humans and animals. They are aerobic, facultatively anaerobic and grow readily in ordinary media. They are motile with peritrichous flagella, break down glucose fermentatively, producing acid and gas or acid only, reduce nitrates to nitrites and form catalase but not oxidase. Within the family, they vary widely in their biochemical and antigenic properties. Most of the genera, which are commensal in the gut, can cause infection in certain circumstances. The exceptions to this are *Salmonella* and *Shigella,* which are always pathogenic.

Classification

Currently, the enteric bacilli are grouped based on similar DNA base compositions and a number of common morphological and biochemical properties.

Two widely used systems for the classification of Enterobacteriaceae are:

1. **Bergey's** classification
2. **Edwards–Ewing** classification (Table 30.1)
3. **Kauffmann and White's** classification (applied only to *Salmonella*)

The species are further classified into types—**biotypes, serotypes, bacteriophage types and colicin types.**

Table 30.1 Enterobacteriaceae: classification by Edwards and Ewing

Tribe	Genus	Species examples
Escherichiae	*Escherichia,* *Shigella*	*E. coli* *S. dysenteriae, S. boydii, S. flexneri* and *S. sonnei*
Edwardsiellae	*Edwardsiella*	*E. tarda*
Salmonellae	*Salmonella*	*S. typhi, S. paratyphi A* and *B*
Citrobacteriaceae	*Citrobacter*	*C. freundii, C. diversus*
Klebsielleae	*Klebsiella* *Enterobacter* *Serratia* *Hafnia* *Pantoea*	*K. pneumoniae* *E. cloacae* *S. marcescens* *H. alvei* *P. agglomerans*
Proteeae	*Proteus* *Providencia* *Morganella*	*P. vulgaris, P. mirabilis* *P. stuartii* *M. morganii*
Yersinieae	*Yersinia*	*Y. enterocolitica*
Erwinieae	*Erwinia*	*E. herbicola*

TRIBE ESCHERICHIAE

ESCHERICHIA COLI

Clinical Case 1

A 25-year-old woman complained of frequent urination and dysuria for two days followed by fever with chills for the past 24 hours. Microscopic examination of her urine showed pus cells of >1 per high-power field in an uncentrifuged specimen, and no casts. Urine culture by semi-quantitative method grew >10^5/CFU/mL of lactose-fermenting colonies. These were identified as E. coli, *which was susceptible to ciprofloxacin, nitrofurantoin, gentamicin, piperacillin and tazobactam. The patient was treated with ciprofloxacin.*

This genus is named after Escherich (1885), who was the first to describe it.

Presently, one major group, *Escherichia coli* is the recognised human enteric flora.

Unlike other coliforms, *E. coli* is a parasite living only in the human or animal intestine. Voided in feces, it remains viable in the environment only for a few days**. Detection of thermotolerant *E. coli* in drinking water indicates fecal contamination of the water.**

Morphology

E. coli is a gram-negative, straight rod measuring 1–3 μm × 0.4–0.7 μm, arranged singly or in pairs (Fig. 30.1). It is motile by peritrichate flagella. Capsules and fimbriae are found in some virulent strains.

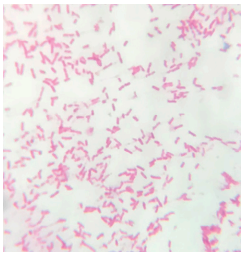


Fig. 30.1 *E. coli* gram-negative straight rods
(*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Antigenic Structure

Serotyping or antigenic typing of *E. coli* is based on three antigens: **somatic antigen O, flagellar antigen H** and **capsular antigen K**. In addition, it also has fimbrial or F antigens. The antigenic pattern of a strain is based on the numerical type of the antigen it carries (e.g., O111: K58:H2).

- **O antigen (somatic antigen)** Around 170 types of heat-stable, lipopolysaccharide O antigens have been recognised. O antigens are associated with the virulence of the organism. The normal colon strains belong to the '**early**' **O groups** (1, 2, 3, 4, etc.), while the enteropathogenic strains belong to the '**later**' **O groups** (26, 55, 86, 111, etc.).
- **H antigen (flagellar antigen)** H antigens are more specific since cross-reaction between other members is not common. 75 H antigens have been recognised so far.

- **K antigen (capsular antigens)** About 100 K antigens have been recognised till date. This is an acidic polysaccharide antigen located in the **envelope** or microcapsule. K antigens are currently classified into two groups, I and II.

Virulence Factors

Two types of virulence factors have been recognised in *E. coli:*

1. Surface antigens

- **O antigen** (somatic lipopolysaccharide) It exerts **endotoxic activity**. It also protects the organism from phagocytosis and the bactericidal effects of complements. The envelope or K antigens also offer protection against phagocytosis.
- **KI envelope antigen** Strains causing neonatal meningitis and septicemia carry this antigen, which **resembles the group B antigen of meningococci.**
- **F antigens** Fimbriae are important **virulence factors.** They are heat-labile and get detached when the organism is heated to 100°C. Plasmid-coded fimbriae are found only in small numbers and mediate **mannose-resistant hemagglutinins**. **P fimbriae,** seen in uropathogenic strains, bind specifically to the P blood group substance on human erythrocytes and uroepithelial cells. Fimbriae are important in initial attachment and colonisation.

Colonisation factor antigens (CFA) occur in enterotoxigenic *E. coli,* causing human diarrhea.

2. Toxins

E. coli produces two kinds of exotoxins: hemolysins and enterotoxins.

Hemolysins

Hemolysins do not appear to be relevant in pathogenesis though they are produced more commonly by virulent strains than by avirulent strains. **CNF1** (cytotoxic necrotising factor-1) and **siderophores** are virulence factors in uropathogenic *E. coli* and are important components of biofilm

production and adhesion.

Enterotoxins

Enterotoxins are important in the pathogenesis of diarrhea. Three distinct types of *E. coli* enterotoxins have been identified: **heat-labile toxin (LT), heat-stable toxin (ST)** and **verotoxin (VT),** also known as **Shiga-like toxin (SLT).** Methods of detection of the two toxin components are shown in Table 30.2.

Table 30.2 Methods for detection of ETEC-labile (LT) and ETEC-stable (ST) enterotoxins (ETEC=Enterotoxigenic *E. coli*)

In vitro assays	LT	ST
1. *Tissue culture tests*		
Rounding of Y1 mouse adrenal cells	+	–
Elongation of Chinese hamster ovary (CHO) cells	+	–
2. *Serological tests*		
ELISA	+	ST ELISA with monoclonal antibody
Passive agglutination tests, passive immune hemolysis, precipitin (Eiken's) test	+	–
3. *Genetic tests*		
DNA probes	+	+

i) **Heat-labile toxin (LT)** of *E. coli* was discovered in 1956 by De and colleagues in isolates from adult diarrhea cases in Kolkata. *E. coli* LT resembles the cholera toxin in its structure, antigenic properties and mode of action. It is a complex of polypeptide subunits—each unit consisting of **one subunit A** (A for **active**) and **five subunits B** (B for **binding**). The toxin binds to the GM1 ganglioside receptor on intestinal epithelial cells by means of subunit B, following which, subunit A is activated to yield two fragments: A1 and A2. The A1 fragment activates adenylyl cyclase in the enterocyte to form cyclic

adenosine 5' monophosphate (cAMP), leading to the increased outflow of water and electrolytes into the gut lumen, with consequent watery diarrhea.

ii) **Heat-stable toxin (ST)** of *E. coli* was first identified in 1970 and comprises low-molecular-weight polypeptides which are poorly antigenic. Two types of STs are known:

- **ST_A**, which acts by activation of cyclic guanosine monophosphate (cGMP) in the intestine.
- **ST_B,** the mode of action of which is not entirely understood; what is known is that it is not through cAMP or cGMP.

ST_A and ST_B genes are not carried on the same plasmid.

iii) ***E. coli* verocytotoxin or verotoxin** is so named because of its cytotoxic effect on Vero cells (cell line derived from African green monkey kidney cells). It is also known as Shiga-like toxin (SLT) because of its similarity to *Shigella dysenteriae* type 1 toxin, in its physical, antigenic and biological properties. It acts by inhibiting protein synthesis. Besides cytotoxicity in Vero and HeLa cells, VT also shows enterotoxicity in rabbit ileal loops like the Shiga toxin. VT is also composed of A and B subunits. The genes appear to be phage-encoded. An antigenically different VT, called VT_2 has been identified, which is not neutralised by the Shiga antitoxin, unlike VT_1.

Clinical Infections

Four main types of clinical syndromes are caused by *E. coli*:

I. Urinary tract infection
II. Diarrhea
III. Septicemia, neonatal sepsis and neonatal meningitis
IV. Pyogenic infections

I. Urinary tract infection

E. coli and other coliforms account for the large majority of naturally acquired urinary tract infections (UTI). The *E. coli* serotypes commonly responsible for community-acquired UTI (*Clinical case* 1) are those

normally found in the gut of the person, O groups 1, 2, 4, 6, 7, etc.

About 5–7 per cent of pregnant women have been reported to have **asymptomatic bacteriuria,** which, if undetected and untreated, may lead to symptomatic infection later in pregnancy, pyelonephritis and hypertension, leading to prematurity and perinatal death of the fetus.

Strains carrying K antigens are more commonly responsible for pyelonephritis, while most isolates from cystitis lack K antigens. **The P-pili-positive *E. coli* are generally uropathogenic.**

Laboratory diagnosis

Significant bacteriuria

Bacteriological diagnosis of UTI is done by demonstrating '**significant bacteriuria**' using semi-quantitative cultures (**developed by Kass**) (Fig. 30.2). This is based on the fact that normal urine is sterile, but during voiding may become contaminated with genital commensals. The counts in the contaminated urine would be lower than that caused in the case of a UTI. A count of 100,000 bacteria per mL is considered 'significant' (suggesting infection) in voided urine.

Fig. 30.2 Semi-quantitative culture of urine with $>10^5$ CFU/mL *E. coli* colonies on CLED (cysteine-, lactose- and electrolyte-deficient) medium (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

1. Specimen

- **Freshly voided urine** Following urogenital toilet, a clean-catch midstream sample of urine is collected.
- **Suprapubic aspiration** In obstructive uropathy, when voiding is not possible, a suprapubic aspiration may be done.
- **Catheterised patients** Urine culture from catheterised patients is not recommended, except immediately after introducing a catheter; urine may be collected from the port.

Counts of 10,000 bacteria or less per mL are due to contamination during

voiding and are not significant unless the patient is on antibiotic treatment or diuretics. In gram-positive organisms like *S. aureus*, lower counts may be significant.

2. **Screening test for UTI** Several screening techniques have been introduced for the rapid presumptive diagnosis of significant bacteriuria:

i) **Griess nitrite test** The presence of nitrite, detectable by a simple colourimetric test, indicates the presence of nitrate-reducing bacteria in urine; normal urine does not contain nitrites.

ii) **Catalase test** The presence of catalase as evidenced by frothing on addition of hydrogen peroxide indicates bacteriuria.

iii) **Triphenyl tetrazolium chloride (TTC) test** This is a dye reduction test signifying respiratory activity of growing bacteria causing urinary tract infection.

iv) **Microscopic demonstration** The bacteria can be demonstrated on Gram-stained films of uncentrifuged urine.

v) **Dip slide culture methods** Agar-coated slides are immersed in urine or even exposed to the stream of urine during voiding. They are incubated and the growth estimated by colony counting or by colour change of indicators in the medium.

3. **Localisation of UTI** This is based on the assumption that bacteria coated with specific antibodies are present in the urine only when the kidneys (upper UTI) are infected and not when the infection is confined to the bladder (lower UTI). Antibody-coated bacteria are detected by immunofluorescence using fluorescent-tagged antihuman globulin.

None of the screening methods is as sensitive or reliable as a culture.

Methods of urine culture

Quantitative culture Serial tenfold dilutions of urine are tested by the pour plate or surface culture methods and the exact bacterial count per millilitre of urine is counted. This, however, is too complicated for routine diagnostic work.

Semi-quantitative techniques These are more convenient and used in routine clinical laboratories. A standard calibrated **loop** is employed to deliver 0.01 mL of sample, which is used to inoculate the plate. One loopful of urine is placed on a non-inhibitory medium (blood agar) and another loopful on an indicator medium (MacConkey). The former gives a quantitative measurement of bacteriuria, while the latter enables presumptive diagnosis of the bacterium. The isolates are identified by their properties.

4. **Cultural characteristics** It grows aerobically and is a facultative anaerobe. The temperature range required for its growth is 10–40°C

(optimum 37°C).

- In **ordinary media,** it grows well. Many pathogenic isolates have polysaccharide capsules. Some strains may occur in the **mucoid** form.
- On **blood agar,** many strains, especially those associated with infection, are hemolytic.
- On **MacConkey medium** (Fig. 30.3), colonies are bright pink due to lactose fermentation.

Fig. 30.3 MacConkey agar with smooth, pink colonies of *Escherichia coli* (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- On **selective media,** growth is largely inhibited, such as **DCA** or **SS agar** used for the isolation of salmonellae and shigellae.
- In **broth**, growth occurs with uniform turbidity and a heavy deposit, which disperses completely on shaking.

5. **Biochemical reactions** Glucose, lactose, mannitol, maltose and several other carbohydrates are fermented with the production of acid and gas. Typical strains do not ferment sucrose.

IMViC test Four biochemical tests widely employed in the classification of Enterobacteriaceae are (i) indole, (ii) methyl red (MR), (iii) Voges–Proskauer (VP) and (iv) citrate utilisation, generally referred to by the mnemonic IMViC.

The details of biochemical reactions of *E. coli* along with other Enterobacteriaceae are given in Table 30.3.

Table 30.3 Enterobacteriaceae: Important distinguishing features of the genera encountered in clinical samples

Species or common name	Lancefield group	Hemolysis	Habitat in human hosts	Laboratory tests	Common diseases caused
S. pyogenes	A Griffith's M-types (80) Nephritogenic pyoderma: M types 2, 49, 57, 59, 60, and 61	Beta	Throat, skin	Bacitracin-sensitive; PYR test-positive; Ribose not fermented	Upper respiratory tract infections, impetigo, pyoderma, Neonatal meningitis, septicemia Sequelae: Rheumatic fever glomerulonephritis
S. agalactiae	B	Beta	Female genital tract, rectum	cAMP test, hippurate hydrolysis	Neonatal infections: meningitis or septicemia Adults: Sepsis, wound infections
S. equisimilis	C	Beta	Throat	Ribose- and trehalose-fermented	
S. anginosus	ACGF and untypable	Beta (alpha or non-hemolytic gamma)	Throat, colon, female genital tract	Bacitracin-resistant, PYR-negative; minute colony variants of other groups	
Enterococcus sp. (*S. faecalis* and other enterococci)	D	Non-hemolytic	Colon, female genital tract throat	Growth in 6.5% NaCl; PYR-positive	Urinary tract infections, endocarditis, suppurative infections
Non-enterococcal Group D species (*S. bovis*)	D	Non-hemolytic	Colon	No growth in 6.5% NaCl	Endocarditis (*S. sanguinis*); dental caries (*S. mutans*)
Viridans streptococci (many species)	Not typed	Alpha	Mouth, colon, female genital tract	Optochin-resistant, species classification on biochemical properties	Infective endocarditis

(d = results different in different species or strains)
Important exceptions:
[1]*S. typhi* does not produce gas from sugars.
[2]*S. sonnei* ferments lactose and sucrose late.

Antimicrobial susceptibility test *E. coli* and other common urinary pathogens develop drug resistance frequently. Isolates are often resistant to multiple antibiotics, which are transferable.

Treatment

Treatment of uncomplicated UTI caused by antibiotic-susceptible *E. coli* is with cotrimoxazole, 500/125 mg BD for 3–5 days or ciprofloxacin, 500 mg BD for 3–5 days.

However, treatment would depend on the susceptibility pattern of the clinical isolate.

II. Diarrhea

In 1945, Bray established the causative role of a specific type of *E. coli* (subsequently recognised as type 0111) during a hospital outbreak of childhood diarrhea in London. There are six diarrheagenic *E. coli* which are

as follows:

1. **Enteropathogenic *E. coli* (EPEC)** These have been associated mainly with diarrhea in infants and children, usually occurring as institutional outbreaks. They occasionally cause sporadic diarrhea in children and less often, in adults. EPEC are identified by their O antigens (e.g., 026:B6, 055:B5, 0111:B4, etc.).

The pathogenesis of EPEC diarrhea is not fully understood. EPEC are non-invasive and do not produce enterotoxins. In infantile enteritis, a plasmid-encoded protein, EPEC adherence factor (**EAF**), has been incriminated in adherence. Another mechanism is by adherence to the enterocyte membrane. This activity is regulated by chromosomally coded locus of enterocyte effacement (**LEE). This is a pathogenicity island by an intimin protein coded for by *eae* gene**. This results in the disruption of the brush border microvilli.

2. **Enterotoxigenic *E. coli* (ETEC)** Two epidemiological types are associated with this:

i) **Endemic in developing countries** of the tropics, seen in all age groups. Its severity varies from mild watery diarrhea to a fatal disease indistinguishable from cholera.

ii) **Traveller's diarrhea** seen in persons from non-endemic areas visiting endemic areas. ETEC serotypes responsible for diarrhea are mostly 06, 08, 015, 025, 027 and 0167.

It primarily adheres to intestinal mucosa by fimbriae called **colonisation factor antigens**, of which several types have been identified (CFA I, II, III and IV). **ETEC produce enterotoxins which may be either LT or ST or both.**

3. **Enteroinvasive *E. coli* (EIEC)** These resemble the **Alkalescens–Dispar Group**. They have been termed enteroinvasive *E. coli* because they have the capacity to invade interstitial epithelial cells as seen in shigellosis. This ability to penetrate cells is determined by a large plasmid, the detection of which can also be a diagnostic test. **EIEC strains belong to serogroups 028 ac, 0112 ac, 0124, 0136, 0143, 0114, 0152** and **0154.**

Molecular serotyping of clinical isolates is presently used to diagnose EIEC infection. HeLa or HEp-2 cell invasion in culture can also be used as a diagnostic test.

4. **Enterohemorrhagic *E. coli* (EHEC)** These strains produce two potent toxins, verocytotoxin (VT) or Shiga-like toxin (SLT). They can give rise to fatal hemorrhagic colitis and hemorrhagic uremic syndrome (HUS), particularly in young children and the elderly. The primary target appears to be the vascular endothelial cells. This may explain the pathogenesis of HUS, in which a characteristic renal lesion is **capillary microangiopathy**. *E. coli* serotype O157:H7 or rarely, O26:H1 are associated with EHEC diarrhea and its complications.

Laboratory diagnosis of VTEC diarrhea can be made by detecting VT in feces directly or in culture isolates. Real-time PCR with specific DNA probes for the VT_1 and VT_2 genes can be done. VT can also be detected by its cytotoxic effects on Vero or HeLa cells. Most VTEC strains belong to the serotype O157:H7, which does not ferment sorbitol, hence the use of sorbitol MacConkey medium helps in screening for O:157 VTEC.

5. **Enteroaggregative *E. coli* (EAEC)** Associated with persistent diarrhea, these appear aggregated in a 'stacked brick' formation on HEp-2 cells or glass. While most of them are O-untypable, many are H-typable.

6. **Diffusely adherent *E. coli* (DAEC)** These strains are defined by a pattern of diffuse adherence (DA), in which the bacteria uniformly cover the entire cell surface. This is unlike the EPEC which have localised adherence. DEAC are less well established as pathogens and may cause diarrhea in children above 12 months of age.

III. Pyogenic infections

E. coli are the most common cause of intra-abdominal infections such as peritonitis and abscesses. **They are an important cause of neonatal meningitis.**

IV. Septicemia

Bloodstream invasion by *E. coli* may lead to fatal conditions like septic shock and systemic inflammatory response syndrome (SIRS).

Uro-sepsis is an emerging complication of urinary tract infection among immunocompromised individuals, especially diabetics and the elderly. This is detected by isolating the same organism from the urine and blood of the patient and noting the similarity in the antibiotic susceptibility pattern. They generally respond to broad-spectrum antibiotics.

As *E. coli* commonly show multiple drug resistance, antibiotic sensitivity, testing of strains is important in treatment**.** Multidrug-resistant (MDR) *E. coli* including isolates producing extended-spectrum beta-lactamase (ESBL) have been associated with hospital-acquired UTI, catheter-associated UTI (CAUTI) and urosepsis.

SHIGELLA

Dysentery is a clinical condition of multiple origin, characterised by the frequent passage of bloodstained, mucopurulent stools. The two common types of dysentery are (i) bacillary and (ii) amoebic. In 1896, Shiga isolated the first member of this genus from epidemic dysentery in Japan.

Clinical Case 2

A 16-year-old boy ate pani-puri from a roadside vendor. Three days later, he developed abdominal cramps with mild fever which was followed by increased frequency of stools with blood and mucus.

He visited the hospital where a stool sample was sent for microscopic examination and culture. The sample showed plenty of pus cells, and the culture was positive for non-lactose-fermenting bacteria on MacConkey agar which agglutinated with antisera for Shigella flexneri*. He was treated with ciprofloxacin, 15 mg/kg of body weight, orally twice daily for 3 days and plenty of oral fluids as supportive therapy.*

Morphology

Shigellae are short, gram-negative rods, about 0.5 × 1–3 μm in size. They are **non-motile,** non-sporing and non-capsulated. Fimbriae may be present.

Classification

Shigellae are classified into four species or subgroups based on a combination of biochemical and serological characteristics. Serotypes are distinguished within the species.

S. dysenteriae (subgroup A)

This species of mannitol non-fermenting bacilli **consists of 12 serotypes**. Type 1 is the bacillus originally described by Shiga (*S. shigae*). It is indole-negative and is the only member of the family that is always **catalase-negative**.

S. dysenteriae type 1 forms a toxin (**Shiga toxin**), the earliest example of an exotoxin produced by a gram-negative bacillus. Three types of toxic activity have been demonstrated in shigella culture filtrates:

1. **Neurotoxicity** The primary site of its action appears to be not the nervous tissue but the blood vessels, mainly those of the central nervous system; neurological effects are secondary.
2. **Enterotoxicity** Two new shigella enterotoxins have been identified, designated **ShET-1 and -2**, the former confined to *S. flexneri* 2a and the latter more widespread. ShET-2 produced by *S. dysenteriae* type 1 forms an exotoxin, ShET, which has the ability to induce inflammation of the epithelial cells by decreased secretion of interleukin-8.
3. **Cytotoxicity** It causes cytopathic changes in cultured Vero cells. This appears to be the **same as verotoxin** (or Shiga-like toxin of *E. coli* – VTEC). The toxin consists of binding (B) and active (A) subunits. Subunit A is divided into two fragments, A1 and A2. Fragment A1 appears to inactivate the host cell's 60S ribosome, interfering with protein synthesis.

S. dysenteriae type 2 (*S. schmitzi*) forms indole and ferments sorbitol and rhamnose. Serotypes 3–7 were described by Large and Sachs in India.

S. flexneri (subgroup B)

This group is named after Flexner and is antigenically the most complex among shigellae. Based on type-specific and group-specific antigens, these bacilli have been classified into six serotypes (1–6) and several subtypes (1a; 1b; 2a, 2b; 3a, 3b, 3c, 4a, 4b, 5a, 5b). In addition, two antigenic 'variants' called X and Y are recognised, which lack the type-specific antigens.

S. boydii (subgroup C)

This group consists of dysentery bacilli that resemble *S. flexneri* biochemically but not antigenically. Eighteen serotypes have been identified.

S. sonnei (subgroup D)

This bacillus, first described by Sonne in Denmark, ferments lactose and sucrose late and is indole-negative. It is antigenically homogeneous but may occur in two forms, phase I and phase II. Organisms in phase II may be isolated from patients but are more common in convalescents and carriers.

S. sonnei causes the mild form of bacillary dysentery. In many cases, the disease may only be a mild diarrhea. However, *S. sonnei* infection continues to be the most common shigellosis in developed countries. *S. sonnei* is serologically homogeneous and is classified by colicin typing into 26 types.

Antigenic Structure

Shigellae possess one or more **'major' antigens** and a large number of '**minor' somatic O antigens.** Some strains possess **K antigens**. The antigenic structure of shigella is simple compared to the complex structure of salmonellae.

There is considerable antigenic sharing between some members of the genus as well as between shigellae and *E. coli*. Common fimbrial antigens may also occur, particularly in *S. flexneri.* It is, therefore, important that the identification of shigellae be made by a combination of antigenic and biochemical properties and not by antigenicity alone.

Resistance

Shigellae are killed at 56°C in one hour and by 1% phenol in 30 minutes. In ice, they last for 1–6 months. *S. sonnei* is in general, more resistant than the other three species.

Pathogenicity

Shigella is the most potent pathogen among the enteric bacteria. Its infective dose is as low as 10–100 bacilli, which are capable of initiating the disease as they survive gastric acidity better than other pathogenic enteric bacteria.

Invasive property The pathogenic mechanisms of shigellae resemble those of enteroinvasive *E. coli*. Organisms bind to M cells and invade the lamina propria, then the neighbouring enterocytes from the bottom (basolateral surface). Bacteria grow and induce actin polymerisation that pushes organisms laterally into the neighbouring cells, where they continue to spread, causing cell death and inflammation. Capillary thrombosis leads to necrosis of patches of epithelium, which slough off, leaving behind **transverse superficial ulcers.** Bacteremia may occur in severe infections, particularly in malnourished children and in AIDS.

The invasive property of the bacillus can be demonstrated by its ability to penetrate cultured HeLa or HEp-2 cells or by the Congo red binding test.

The invasive property is due to the outer membrane protein responsible for cell penetration. These proteins are called **virulence marker antigens** (VMA). Detection of VMA by ELISA serves as a virulence test for shigellae, similar to enteroinvasive *E. coli*.

Bacillary dysentery This disease has a short incubation period (1–7 days, usually 48 hours).

The main **clinical features** are frequent passage of loose, scanty feces containing blood and mucus, along with abdominal cramps and tenesmus (*Clinical case 2*). Fever and vomiting may be present.

Complications These are most often seen in children and immunocompromised patients like those affected by AIDS (mainly due to *S. dysenteriae* type 1). They may include arthritis, toxic neuritis, conjunctivitis, parotitis and, in children, intussusception.

Hemolytic uremic syndrome may occur as a complication in severe cases due to microangiopathic hemolytic anemia, thrombocytopenia and renal failure.

Epidemiology

Human beings are the only natural hosts for shigellae. Transmission is through:

- Contaminated fingers, flies, food and fomites such as door handles, water taps and lavatory seats
- Water and contaminated food or drink

In young male homosexuals, transmission is as part of the **gay bowel syndrome.**

Laboratory Diagnosis

1. **Specimen** Diagnosis is made by isolating the bacillus from feces. Fresh feces should be inoculated without delay.

Enrichment media like **selenite F broth** or **Salmonella Shigella (SS) broth** can be used for selecting the pathogens though they are better for *Salmonella*.

2. **Microscopy** Plenty of pus cells with RBCs are seen under the

microscope.

3. **Cultural characteristics** They are aerobes and facultative anaerobes, with a growth temperature range of 10–40°C (optimum of 37°C) and pH 7.4. Colonies on MacConkey agar are colourless due to the absence of lactose fermentation. An exception is *S. sonnei,* which ferments lactose late and forms pale pink colonies. On **deoxycholate citrate agar (DCA)** and **xylose lysine deoxycholate (XLD),** they appear as red (alkaline) colonies (Fig. 30.4). They are further identified by biochemical reactions.

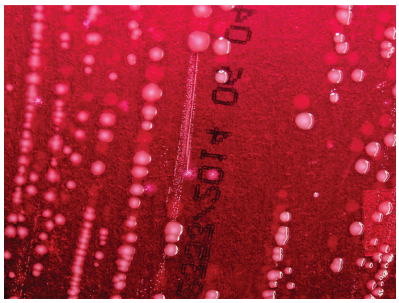


Fig. 30.4 Red colonies of *Shigella sonnei* on XLD agar (*Source:* Public Health Image Library, ID 17191 / Todd Parker, Ph.D., Assoc Director for Laboratory Science, Div of Preparedness and Emerging Infections at CDC / CDC)

4. **Biochemical reactions** Catalase is produced by all bacilli of this group except *S. dysenteriae* type 1. Glucose is fermented with the production of acid, without gas, (except Newcastle and Manchester biotypes of *S. flexneri* type 6, and some strains of *S. boydii* types 13 and 14). **Fermentation of mannitol is of importance in classification**; shigellae have traditionally been divided into mannitol-fermenting and non-fermenting species. *S. flexneri, S. boydii* and *S. sonnei* ferment mannitol, **while *S. dysenteriae* does not**. Exceptions are not infrequent. None of them ferment lactose and sucrose except *S. sonnei,* which ferments them late.

5. **Identification by specific antisera** Identification is confirmed by slide agglutination with polyvalent and monovalent antisera.

Treatment

Uncomplicated shigellosis is a self-limiting condition, hence treatment with antibiotics is not recommended in adults.

However, in acute cases, particularly in infants and young children, dehydration has to be corrected promptly. Oral rehydration is adequate in

most cases. The choice of antibiotic is based on the sensitivity of the prevailing strain. Fluoroquinolones (ciprofloxacin) or cotrimoxazole may be given empirically. However, with increasing antibiotic resistance, a sensitivity test must be performed before prescribing a drug.

Control

Control consists essentially of improving personal and environmental sanitation.

Antibiotics have no place in prophylaxis. No effective vaccine is available.

TRIBE SALMONELLAE

SALMONELLA

The genus *Salmonella* causes enteric fever, gastroenteritis and septicemia with or without focal suppuration. It may lead to a carrier state.

The most important member of the genus is *Salmonella* Typhi, the causative agent of typhoid fever. The organism was first discovered by Eberth (1880) in the mesenteric nodes and spleen of fatal cases of typhoid fever and was first isolated by Gaffky (1884).

Salmonellae currently comprise above 2,000 serotypes or species, all of them potentially pathogenic. For practical and clinical purposes, salmonellae may be divided into two groups:

1. **Typhoidal** The enteric fever group, consisting of the typhoid and paratyphoid bacilli that are exclusively or primarily human parasites.
2. **Non-typhoidal** The food poisoning group, which essentially comprises animal parasites but which can also infect human beings, producing gastroenteritis, septicemia or localised infections.

Morphology

Salmonellae are gram-negative rods, about 1–3 × 0.5 μm in size. They are motile with peritrichate flagella (except *S. gallinarum* and *S. pullorum*).

Clinical Case 3

A 10-year-old boy was admitted to the pediatric ward with a history of remittent fever which increased gradually over the previous 10 days. He was admitted with complaints of loss of appetite, pain in the

abdomen and general weakness. On examination, he was found to have a temperature of 102°F with pallor and coated tongue, mild splenomegaly and abdominal tenderness. His blood culture was positive for Salmonella Typhi with a negative Widal test. The isolate was resistant to ciprofloxacin and so he was treated with ceftriaxone. He responded after 10 days of therapy.

Classification and Nomenclature

Kauffmann–White scheme Salmonella have been named and identified using the Kauffmann–White scheme.

- Inclusion in the genus is based on common biochemical properties.
- Classification within the genus is on the basis of antigenic characterisation (Table 30.4).

Table 30.4 Kaufmann–White scheme: Illustrative examples*

Sero-groups	Serotype	Antigen O		Antigen H
		Phase I	Phase II	
A	*S. paratyphi A*	1, 2, 12	a	–
B	*S. paratyphi B*	1, 4, 5, 12	b	1, 2
	S. typhimurium	1, 4, 5, 12	i	1, 2
	S. chester	4, 5, 12	e, h	e, n, x
C1	*S. paratyphi C*	6, 7, (Vi)	c	1, 5
	S. choleraesuis	6, 7	c	1, 5
C2	*S. muenchen*	6, 8	d	1, 2
D	*S. typhi*	9, 12, (Vi)	d	–
	S. enteritidis	1, 9, 12	g, m	–
	S. gallinarum	1, 9, 12	–	–
E1	*S. anatum*	3, B	e, h	1, 6

*As the Kauffman–White scheme was created before modifications in nomenclature, this table has retained the earlier style. However, in the text, current nomenclature is used.

The classification and nomenclature of salmonellae has undergone modification over the years. Modern taxonomical techniques have shown that the species in the genus Salmonella are very closely related in the genetic, phylogenetic and evolutionary sense. Variations in properties such

as antigenic structure, biochemical reactions and host preferences exhibited by different strains can, therefore, be considered as **intraspecies divergences.**

DNA hybridisation studies have shown that there are two species in the genus *Salmonella:*

1. **Species enterica**, which is further divided into six subspecies:
   I – *S. enterica* subsp. *enterica*
   II – *S. enterica* subsp. *salamae*
   IIIa – *S. enterica* subsp. *arizonae*
   IIIb – *S. enterica* subsp. *diarizonae*
   IV – *S. enterica* subsp. *houtenae*
   VI – *S. enterica* subsp. *indica*
   Most human infections are caused by subspecies *enterica* and rarely by *arizonae*.
2. **Species bongori** (earlier subspecies V)

All these species are further divided into more than 2,500 serovars or serotypes. *Salmonella* typhi is written as **Salmonella enterica subspecies enterica serovar Typhi** or in short, *Salmonella* Typhi or *S*. Typhi (the serovar is not written in italics and also starts with a capital letter). The nomenclature system is based on recommendations from the WHO Collaborating Centre.

Antigenic Structure

Salmonellae possess the following antigens, based on which they are classified and identified:

- Flagellar antigen H
- Somatic antigen O
- Surface antigen Vi, found in some species

H antigen This antigen, which is present on the **flagella, is a heat-labile protein**. It is destroyed by boiling or by treatment with alcohol but not by formaldehyde. When mixed with antisera, **H suspensions agglutinate rapidly, producing large, loose, fluffy clumps**. The H antigen is strongly immunogenic and induces antibody formation rapidly and in high titres

following infection or immunisation.

O antigen The somatic **O antigen is a phospholipid–protein–polysaccharide complex** which forms an integral part of the cell wall. It is **identical to endotoxin**. It can be extracted from the bacterial cell by treatment with trichloroacetic acid, as first shown by Boivin (and therefore, called the **Boivin antigen**).

> The O antigen is unaffected by boiling, alcohol or weak acids. When mixed with antisera, O antigen suspensions form compact, chalky, granular clumps. O agglutination takes place more slowly and at a higher optimum temperature (50–55°C) than H agglutination (37°C).

The antibody to the O antigen is cross-reactive while that to the H antigen is a more reliable indicator.

Viantigen Felix and Pitt first described this antigen.

It is analogous to the K antigens of coliforms and envelops the O antigen, as a result of which, strains of *S. Typhi* that have this antigen do not agglutinate with O antiserum.

It is heat-labile; bacilli that are not agglutinable with the O antiserum become agglutinable after boiling or heating at 60°C for one hour due to the loss of this antigen. It is also destroyed by 1 N HCl and 0.5 N NaOH. It is unaffected by alcohol or 0.2% formaldehyde.

Originally observed in *S.* Typhi, the Vi antigen with similar antigenic specificity is present in *S.* Paratyphi C and *S.* Dublin, as well as in certain strains of *Citrobacter* (the Bethesda–Ballerup group). In human volunteer experiments, strains possessing the Vi antigen were found to cause clinical disease more consistently than those lacking it.

The protective efficacy of the Vi antigen is demonstrated by the success of the purified Vi vaccine for typhoid, now in routine use.

Antigenic variations

The antigens of salmonellae undergo phenotypic and genotypic variation:

H → O variation This variation is associated with the loss of flagella. Rarely, salmonellae may lose their flagella by mutation. **A stable non-motile mutant of *S.*Typhi is the 901-O strain which is widely employed for the preparation of O-agglutinable bacterial suspensions**. To obtain a population of motile cells rich in H antigen from such cultures, selection

may be carried out by using Craigie's tube (Fig. 30.5).

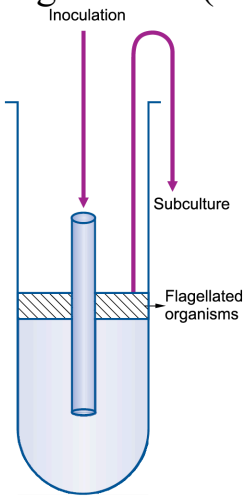


Fig. 30.5 Craigie's tube

Phase variation The flagellar antigens of most salmonellae occur in one of two phases. Phase 1 antigens are either specific for a species or shared by a few species only. Hence it is called the **specific** phase. Phase 2 antigens are widely shared and hence this is called the **non-specific** or **group** phase. Phase 1 antigens are designated as a, b, c, d, etc., and after z, as z1, z2, etc. Phase 2 antigens are far fewer and are designated as 1, 2, etc.

For serotyping of *Salmonella* isolates, it may be necessary to identify the flagellar antigens of both phases. A culture in phase 1 can be converted to phase 2 by passing it through a Craigie's tube containing specific phase 1 antiserum, and the reverse conversion achieved by using phase 2 antiserum.

V → W variation Fresh isolates of *S.* Typhi generally carry a surface layer of Vi antigen that completely masks the O antigen. Such bacilli are agglutinable with the Vi antiserum but not with the O antiserum. This is called the **V form**. After a number of subcultures, the Vi antigen is completely lost. This is called the **W form**. Intermediate stages during the loss of the Vi antigen, when the bacillus is agglutinable with both Vi and O antisera, are called
VW forms.

S → R variation The **s**mooth-to-**r**ough variation is associated with a change in colony morphology and loss of the O antigen and thereby, the virulence.

Resistance

The bacilli are killed at 55°C in one hour or at 60°C in 15 minutes.

- Boiling or chlorination of water and pasteurisation of milk destroys the bacilli.
- In polluted water and soil, they survive for weeks and in ice for months.
- They are killed within five minutes by mercuric chloride (1:500) or 5% phenol.

Pathogenicity

Salmonellae are strict parasites of animals or humans. *S.* Typhi and *S.* Paratyphi A and usually, but not invariably, *S.* Paratyphi B are confined to human beings.

Some salmonellae cause asymptomatic conditions or fatal infections, and sometimes epizootic disease. *S.* Typhimurium and *S.* Enteritidis cause fatal septicemia in rats and mice. Domestic animals, reptiles and birds may harbour species which are host-adapted, e.g., *S.* Abortus-equi is found only in horses, *S.* Abortus-ovis in sheep and *S.* Gallinarum in poultry. Others such as *S.* Typhimurium have a wide host range, affecting animals, birds and humans.

Salmonellae cause the following clinical syndromes in humans:

1. Enteric fever
2. Gastroenteritis or food poisoning
3. Septicemia with or without local suppurative lesions

1. Enteric Fever

The term enteric fever includes typhoid fever caused by *S.* Typhi and paratyphoid fever caused by *S.* Paratyphi A, B and C.

The infection is acquired by ingestion. The infective dose is 10^3 to 10^5 bacilli (greater than *Shigella*). On reaching the gut, the bacilli attach themselves to the microvilli of the ileal mucosa and penetrate to the lamina propria and submucosa. They can invade macrophages, dendritic and epithelial cells. The ability to resist intracellular killing and to multiply within these cells is a measure of their virulence. The genes responsible for the **virulence** for invasion, survival and extraintestinal spread are located

in **Salmonella pathogenicity islands (SPIs)**. Salmonella enter the mesenteric lymph nodes, where they multiply and, via the thoracic duct, enter the bloodstream. Transient bacteremia follows, during which the bacilli are seeded in the liver, gallbladder, spleen, bone marrow, lymph nodes, lungs and kidneys, where further multiplication takes place. Towards the end of the incubation period, there occurs massive bacteremia from these sites of multiplication, heralding the onset of clinical disease.

As bile is a good culture medium for the bacillus, it multiplies abundantly in the gallbladder and is discharged continuously into the intestine, where it involves the Peyer's patches and the lymphoid follicles of the ileum. These become inflamed, undergo necrosis and slough off, leaving behind the **characteristic typhoid ulcers**. Ulceration of the bowel leads to the two major complications of the disease—intestinal perforation and hemorrhage. However, these are no longer observed due to rapid diagnosis and appropriate antibiotic treatment. During the 3–4 weeks that normally constitute the course of the disease, the intestinal lesions undergo healing.

Clinical Course

The incubation period is usually 7–14 days but may be in the range of 3–56 days. The clinical course may vary from mild undifferentiated pyrexia (ambulant typhoid) to a rapidly fatal disease (*Clinical case* 3). The typical features are **step-ladder pyrexia**, with relative bradycardia and toxemia. These features are no longer observed due to the use of antipyretics at the onset of fever.

A soft**, palpable spleen** is a constant finding. Hepatomegaly is also common.

'**Rose spots**' that fade on pressure appear on the skin during the second or third week but are seldom noticeable in dark-skinned patients.

Complications

The most significant complications are intestinal perforation, hemorrhage and circulatory collapse. Some degree of bronchitis or bronchopneumonia is always found. Some develop transient psychoses (**coma vigil**), deafness or meningitis. Cholecystitis, arthritis, abscesses, periostitis, nephritis, hemolytic anemia, venous thromboses and peripheral neuritis are other

complications. Osteomyelitis is a rare sequel.

Convalescence is slow. In about 5–10 per cent of cases, relapse occurs during convalescence. *S.* Paratyphi A and B cause paratyphoid fever which resembles typhoid fever but is generally milder. *S.* Paratyphi C may also cause paratyphoid fever but more often, it leads to frank septicemia with suppurative complications.

Epidemiology

Typhoid fever has been virtually eliminated in the developed countries over the past several decades, mainly as a result of improvements in water supply and sanitation. However, it continues to be endemic in the developing world. *S.* Paratyphi A is prevalent in India and other Asian countries.

Enteric fever is endemic in all parts of India. An incidence of 500–980 per 100,000 population has been reported in different studies varying with age and geographical area. Worldwide, 22 million cases are estimated to occur annually, with 600,000 deaths (highest concentration in Asia)! Paratyphoid B is rare and C, very rare. The disease occurs at all ages but is probably most common in the age group of 5–20 years. Enteric fever due to *Salmonella* Paratyphi A has increased over the last decade in India. The isolates appear to have changing antibiotic resistance pattern.

Carriers

An individual is said to be a carrier if she continues to shed the bacteria in feces up to a year after the initial infection. Patients who continue to shed typhoid bacilli in feces for three weeks to three months after clinical cure are called **convalescent carriers**. Those who shed the bacilli for more than three months but less than a year are called **temporary carriers** and those who shed the bacilli for over a year are called **chronic carriers**.

The bacilli persist in the gallbladder or kidneys and are eliminated in the feces (fecal carrier) or urine (urinary carrier).

Food handlers or cooks who become carriers are particularly dangerous. The best known of such typhoid carriers was Mary Mallon ('**Typhoid Mary**'), a New York cook who, over a period of 15 years, caused at least seven outbreaks, affecting over 200 persons.

Carriers exist for paratyphoid bacilli also. While *S.* Paratyphi A occurs only in human beings, *S.* Paratyphi B can infect animals such as dogs or cows, which may act as sources of human disease.

Typhoid fever occurs in two epidemiological types:

- **Endemic** or **residual typhoid**
- **Epidemic typhoid**, which may occur in endemic or non-endemic areas; typhoid epidemics are water-, milk- or food-borne

Laboratory Diagnosis

Bacteriological diagnosis of enteric fever consists of isolation of the bacilli from the patient and the demonstration of antibodies in his serum (Fig. 30.6).

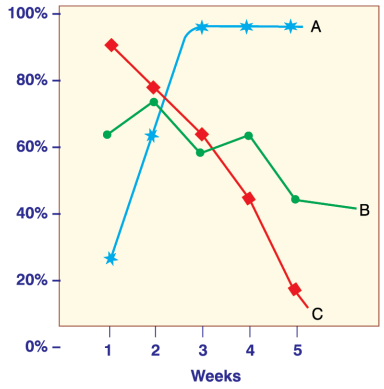


Fig. 30.6 Percent positivity of various laboratory diagnostic tests for typhoid fever during different stages of the disease (weeks 1–5); A. Widal test, B. feces culture and C. blood culture

1. **Specimen** Blood is collected for culture, as is urine and stool in the later part of infection. Serum is obtained for the Widal test.

2. **Blood culture** A positive blood culture is diagnostic. Bacteremia occurs early in the disease, and blood cultures are positive in approximately 90 per cent of cases in the first week of fever. Isolation decreases with increasing duration. Automated blood culture systems have increased the isolation rates and decreased the turnaround time for a positive culture.

3. **Cultural characteristics** Salmonellae are aerobic and facultatively anaerobic and grow on simple media over a pH range of 6–8 and temperature 15–41°C (optimum 37°C). On **MacConkey agar**, they grow as non-lactose-fermenting colonies. On **deoxycholate citrate** media and **XLD** (xylose lysine deoxycholate), colonies show black centres due to production of H_2S (Fig. 30.7). On the **Wilson** and **Blair** bismuth sulphite medium, jet

black colonies with a metallic sheen are formed due to the production of H_2S. *S.* Paratyphi A and other species that do not form H_2S produce green colonies.

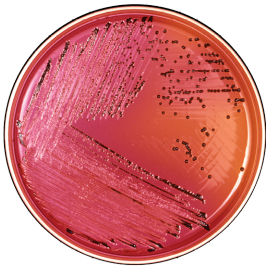


Fig. 30.7 *Salmonella* on xylose lysine deoxycholate (XLD) media
(*Source:* Public Health Image Library, ID 6619 / CDC)

Selenite F and **tetrathionate** broth are commonly employed as enrichment media to isolate *Salmonella* from feces.

4. **Biochemical reactions** Salmonellae ferment glucose, mannitol and maltose, forming acid and gas. An important **exception is *S.* Typhi, which is anaerogenic (does not produce gas).** Lactose, sucrose and salicin are not fermented. Indole is not produced. They are MR-positive, VP-negative and citrate-positive. *S.* Typhi and a few other salmonellae do not grow in Simmons' citrate medium as they need tryptophan as the growth factor. Urea is not hydrolysed. H_2S is produced, except by *S.*Paratyphi A, *S.* Choleraesuis and few other species.

The enteric fever group may be classified biochemically (Table 30.5).

Table 30.5 Biochemical characteristics of typhoid and paratyphoid bacilli

	Glucose	Xylose	d-Tartrate	Mucate
S. Typhi	A	d	A	d
S. Paratyphi A	AG	–	–	–
S. Paratyphi B	AG	AG	–	AG
S. Paratyphi C	AG	AG	AG	–

5. **Serotyping by agglutination** Agglutination of a culture suspension with specific antisera indicates that the isolate belongs to that particular serotype, e.g., identity as *S.* Typhi is established by agglutination with the flagellar antiserum (anti-D serum).

Quite often, fresh isolates of *S.* Typhi are in the V form and do not agglutinate with the O antiserum.

Such strains may be tested for agglutination against the anti-Vi serum. Alternatively, the growth is scraped off in a small amount of saline, boiled for 20 minutes and tested for agglutination with the O antiserum.

Where the isolate is a non-typhoid *Salmonella* (which produces gas from sugars), it is tested for agglutination with O and H antisera for groups A, B and C. Identification of unusual serotypes is done at the **National Salmonella Reference Centre** at the Central Research Institute, Kasauli. The reference centre for salmonellae of animal origin is the Indian Veterinary Research Institute, Izatnagar.

Clot culture Blood is withdrawn from the patient into a sterile test tube and allowed to clot. The clot is broken up and added to a bottle of bile broth. The incorporation of streptokinase (100 units per mL) in the broth facilitates the lysis of the clot. Clot cultures yield a higher rate of isolation than blood cultures.

Feces culture Salmonellae are shed in feces throughout the course of the disease and even in convalescence with varying frequency. Hence fecal cultures are almost as valuable as blood cultures in diagnosis.

Fecal samples are plated directly on **MacConkey, DCA** or **XLD** and **Wilson–Blair media**. For enrichment, specimens are inoculated into one tube each of selenite and tetrathionate broth, and incubated for 12–18 hours before subculture onto plates.

Urine culture Salmonellae are shed in urine irregularly and infrequently. Hence urine culture is less useful than the culture of blood or feces.

Other materials for culture Isolation may be obtained from several other sources but none of these methods are usually employed.

- **Bone marrow** culture is valuable as it is positive in most cases, even when blood cultures are negative.
- **Bile** obtained by duodenal aspiration is usually positive and may be employed for the detection of carriers.
- Other materials which may yield isolation at times are rose spots, pus from suppurative lesions, CSF and sputum. At autopsy, cultures may be obtained from the gallbladder, liver, spleen and mesenteric lymph nodes.

6. Serology

Widal test This is a test for the measurement of H and O agglutinins for typhoid and paratyphoid bacilli in the patient's sera.

Procedure Equal volumes (0.4 mL) of serial dilutions of the serum (from 1/10 to 1/640) and the H and O antigens are mixed in Kahn tubes and incubated in a water bath at 37°C overnight. Some recommend incubation at 50–55°C for two hours, followed by overnight incubation at room temperature. Control tubes containing the antigen and normal saline are set up to check for autoagglutination. The agglutination titres of the serum are read. H agglutination leads to the formation of loose, cotton-woolly clumps, while O agglutination is seen as a disc-like pattern at the bottom of the tube. In both, the supernatant fluid is rendered clear.

A slide test is available commercially and has been introduced in diagnostic laboratories. This procedure uses a coloured antigen. A drop of the antigen is added to a drop of the patient's sera on a slide. The O, H, AH and BH antigens are added in separate reaction circles. If the reaction mixture agglutinates in any one of the mixtures, further dilutions are done either on the slides as per the manufacturer's instructions or by the standard tube method as already described.

Interpretation of results The results of the Widal test should be interpreted taking into account the following:

- The results of a **single test** should be interpreted with caution. It is necessary to obtain information on the distribution of antibody levels in 'normal population' in different areas to determine the cut-off titre.
- *Endemicity of the disease* As typhoid is an endemic in most parts of the country, titres of TO > 80 and TH >160 are taken as indicative of early infection. In non-endemic areas, lower titres may indicate infection.
- Increase in TH, AH and BH may indicate vaccination.
- The agglutination titre will depend on the **stage** of the disease. Agglutinins usually appear by the end of the first week, so blood taken earlier may give a negative result. The titre increases

steadily till the third or fourth week, after which it declines gradually.

- Demonstration of a **rise in titre** of antibodies by testing two or more serum samples is more meaningful than a single test. If the first sample is taken late in the disease, a rise may not be demonstrable. Instead, a fall in titre may be seen in some cases.
- Agglutinins may be present on account of prior disease, inapparent infection or **immunisation.**

H agglutinins persist longer than **O agglutinins**. Serum from an individual immunised with the TAB vaccine will have antibodies to *S.* Typhi and *S.* Paratyphi A and B, while in the case of infection, antibodies will be seen only against the infecting species.

Persons who have had prior infection or immunisation may develop an **anamnestic response** during an unrelated fever.

IgM detection kits
They can be useful in the diagnosis of infection in the initial weeks in a primary infection when a single serum sample is available. They detect IgM antibodies to lipopolysaccharide or outer membrane protein antigens.

Cases treated early with **antibiotics** may show poor agglutinin response.

PCR-based tests These are also sensitive but not widely used due to the cost.

Other laboratory tests White cell count is useful. Leucopenia with relative lymphocytosis is generally seen. Eosinophils are said to be absent but in the tropics, with a high incidence of helminthic infestation, eosinophils are usually present.

7. **Diagnosis of carriers** The detection of carriers is important for epidemiological and public health purposes. Laboratory tests are also useful in screening food handlers and cooks to detect the carrier state.

Identification of fecal carriers is by **isolation** of the bacillus from feces (often, several cultures are required as there may be intermittent shedding) or from bile. For the detection of urinary carriers, repeated urine cultures should be carried out.

Demonstration of antibodies to Vi antigens indicates the carrier state. While this is useful as a screening test, confirmation should be made by

culture.

A technique of isolating salmonellae from sewage is by **filtration through Millipore membranes** and culturing the membranes on highly selective media such as Wilson and Blair media.

Typing Methods

Bacteriophage typing

Phage typing helps in tracing the source of epidemics and provides information on the trends and patterns in the epidemiology of typhoid at the local, national and international levels. As phage typing of *S.* Typhi depends on the presence of Vi antigens, a proportion of strains (Vi negative) will be **untypeable**. **Among *S.* Typhi phage types, A and E1 are more common in India, while among the *S.* Paratyphi A phage, types 1 and 2 are the most common.** The **National Salmonella Phage Typing Centre** for India is located at the Lady Hardinge Medical College, New Delhi.

Molecular methods

Currently, more discriminating genotyping methods have been employed for epidemiological typing, which include the following:

- Pulse field gel electrophoresis (PFGE)
- Multilocus enzyme electrophoresis
- IS-200 profiling
- Random amplified polymorphic DNA (RAPD) analysis

Newer diagnostic tests

As an alternative to the Widal test, which has several limitations, advanced diagnostic tests are now used. These are:

- IgM O9 antibodies can be detected in patients within a few minutes by a tube test, e.g., IDL Tubex.
- Another rapid serological test, Typhidot, takes three hours to perform. It can detect specific IgM and IgG antibodies against a 50 kD antigen of *S*. Typhi. A newer version of the test can detect specific IgM antibodies only.

Prevention

Typhoid fever can be effectively controlled by improvements in sanitation and provision of protected water supply. Many developed countries have been able to eliminate the risk of typhoid by implementing these measures; however, occasional outbreaks do appear due to unforeseen lapses.

Vaccines

TAB vaccine The TAB vaccine that was used earlier contained *S.* Typhi, 1,000 million and *S.* Paratyphi A and B, 750 million each per mL, killed by heating at 50–60°C and preserved in 0.5% phenol. However, on account of the low incidence of *S. Paratyphi* B infections in the recent times, TAB is no longer in use.

Dose The vaccine is given in two doses of 0.5 mL each, subcutaneously at an interval of 4–6 weeks. In non-endemic areas, vaccination is recommended for troops and medical and paramedical personnel. In endemic areas, vaccination is recommended for all children in whom a single dose might give adequate protection and is maintained for several years by the booster effect of repeated natural subclinical infections. **The killed vaccines do not provide cell-mediated immunity.**

Live oral vaccine A live oral vaccine has been introduced after successful field trials. This vaccine (**typhoral**) is a stable mutant of *S.* Typhi strain Ty2 1a, lacking the enzyme UDP-galactose-4-epimerase (Gal E mutant). On ingestion, it initiates infection but 'self-destructs' after four or five cell divisions, and therefore, cannot induce any illness.

Dose The vaccine is an enteric-coated capsule containing 10^9 viable lyophilised mutant bacilli. One capsule is taken orally, an hour before food, with a glass of water or milk, on days 1, 3 and 5. **No antibiotic should be taken during this period.**

Vi vaccine The injectable vaccine (**Typhim-Vi**) contains purified Vi polysaccharide antigen (25 mg per dose) from *S.* Typhi strain Ty2. It is given as a single subcutaneous or intramuscular injection, which causes only minimal local reaction.

Both the oral and Vi vaccines are recommended only for those over five years of age; the same dosage is recommended for children and adults. Protection is stated to commence 2–3 weeks after administration and lasts

for at least three years, after which, a booster must be given. Both the vaccines are effective, however, their relatively high cost hinders wider use.

Natural immunity Typhoid bacilli are primarily intracellular parasites, hence cell-mediated immunity develops during the course of infection. CMI is common in populations in typhoid endemic areas. The killed vaccines currently used do not stimulate CMI.

Treatment

Chloramphenicol was the sheet anchor against typhoid until the 1970s, when resistance became common. Ampicillin, amoxicillin and cotrimoxazole were the other drugs found useful in the treatment of typhoid fever until recently—current strains show resistance to these also. At present, ciprofloxacin is the drug of choice or, in case of resistance, **ceftriaxone** (1–2 g/day). Some centres use **azithromycin** for better patient compliance.

Carriers A combination of antibacterial therapy along with the vaccine has been tried for the eradication of the carrier state. This combination has also been used to prevent relapses. Elimination of the persistent carrier state may, on rare occasions, require cholecystectomy, pyelolithotomy or nephrectomy.

Drug Resistance

Though occasional resistant strains had been identified in the laboratory, resistance to chloramphenicol first emerged in 1972, in Mexico and in Kerala (India).

In India, chloramphenicol-resistant typhoid fever first appeared in epidemic form in Calicut (Kerala) in early 1972 and was confined to Kerala till 1978. Subsequently, strains carrying resistant plasmids appeared in other parts of India. Though strains were resistant to chloramphenicol, they were initially sensitive to ampicillin, amoxicillin, cotrimoxazole and furazolidone. By the late 1980s, resistance to many or all of these drugs began to spread to most parts of India. The drugs useful in the treatment of such multiresistant isolates were the higher classes of fluoroquinolones (such as ciprofloxacin, pefloxacin, ofloxacin) and the third-generation cephalosporins (such as ceftriaxone and cefotaxime). However, resistance

to ciprofloxacin has emerged in several parts of the country. On the other hand, *Salmonella* Typhi and Paratyphi A are regaining their sensitivity to chloramphenicol.

2. Salmonella Gastroenteritis (Non-Typhoidal Salmonella)

Salmonella gastroenteritis (more appropriately, enterocolitis) or food poisoning is generally a zoonotic disease, the source of infection being animal products. It may be caused by non-typhoidal salmonellae. In most parts of the world, *S.* typhimurium is the most common species. Some other common species are *S.* enteritidis, *S.* haldar, *S.* heidelberg, *S.* agona, *S.* virchow, *S.* seftenberg, *S.* indiana, *S.* newport and *S.* anatum.

Sources of Infection

Human infection results from the ingestion of contaminated food, mainly poultry (including eggs and egg products), meat, milk and milk products. The role of human carriers is minimal compared to the magnitude of infection from animals. Salads and other uncooked vegetables may cause infection if contaminated by manure or by handling. Food contamination may also result from the droppings of rats, lizards or other small animals. **Gastroenteritis can occur without food poisoning, as in cross-infection in hospitals.**

Pathogenesis

A short incubation period of 24 hours or less, with diarrhea, vomiting, abdominal pain and fever are the main clinical features. Severity of the condition may vary from the passage of one or two loose stools to an acute cholera-like disease. It usually subsides in 2–4 days but in some cases, a more prolonged enteritis develops, with the passage of mucus and pus in feces, resembling dysentery. In a few, typhoidal or septicemic type of fever may develop.

Laboratory Diagnosis

The etiological diagnosis is confirmed by the culture of feces. In outbreaks

of food poisoning, the causative organism can be isolated from the article of food.

Control is by adhering to personal and food hygiene.

Salmonella food poisoning is also a problem in developed nations. This is due to the differences in food habits (eating salads and undercooked meat and meat products), large scale food production, packaging, storage and marketing.

Treatment

For uncomplicated, non-invasive Salmonella gastroenteritis, antibiotics should not be used. Not only do they not hasten recovery, they may also increase the period of fecal shedding of the bacilli. However, for serious invasive cases, antibiotic treatment is needed.

3. Salmonella Septicemia

Certain salmonellae, *S.* Choleraesuis in particular, may cause septicemic disease with focal suppurative lesions, such as osteomyelitis, deep abscesses, endocarditis, pneumonia and meningitis. The case fatality may be as high as 25 per cent. Salmonellae may be isolated from blood or pus from the suppurative lesion.

In India, several hospital outbreaks of neonatal septicemia caused by multiresistant salmonellae have occurred in recent years. Mortality in neonates is very high unless early treatment is started with antibiotics to which the infecting strain is sensitive.

Treatment is as for enteric fever.

Non-typhoid salmonellosis is a frequent infectious complication in systemic lupus erythematosus (SLE). The extraintestinal manifestations and sepsis are more frequent in SLE than in simple gastroenteritis.

Antibiotic resistance in *Salmonella*

Multiresistant salmonellae have now become important agents of hospital cross-infections. **R factors conferring multiple drug resistance** were first observed in

S. Typhimurium phage type 29 in England in the 1960s. They have become widely disseminated among salmonellae.

There are two categories of drug resistance in *S.* Typhi:

i) Resistance to antibiotics such as chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole (MDR strains).
ii) Resistance to the fluoroquinolone drugs, which may be total or partial. The so-called **nalidixic-acid-resistant *S*. Typhi** (NARST) was a marker of reduced susceptibility to fluoroquinolones compared to nalidixic-acid-sensitive strains.

Vaccines do not protect against Salmonella gastroenteritis or Salmonella septicemia.

Reference centres for Salmonella in India
National Salmonella and Escherichia Centre (NSEC) in Central Research Institute, Kasauli, is the national reference centre for the serotyping of *Salmonella* and *Escherichia coli* for epidemiological studies. It was established in collaboration with Public Health Laboratories, Colindale, UK, and WHO.

TRIBE YERSINIA

YERSINIA ENTEROCOLITICA

Yersinia is a gram-negative bacterium of the family Enterobacteriaceae. Two species are incriminated in human infection: i) *Y. enterocolitica* and ii) *Y. pseudotuberculosis.*

Y. enterocolitica causes an acute bacterial enteric disease characterised by febrile diarrhea and enterocolitis that is indistinguishable from *Salmonella* or *Shigella* gastroenteritis. *Y. enterocolitica* and *Y. pseudotuberculosis* cause acute mesenteric lymphadenitis that mimics appendicitis.

Human diseases caused by *Yersinia* are of three types:

1. The first occurs in young children as **self-limiting gastroenteritis** or **enterocolitis** which may be inflammatory or non-inflammatory.
2. The second is **mesenteric adenitis** and **inflammatory terminal ileitis** in older children, which may mimic appendicitis.
3. The third is a **systemic disease** typically seen in adults, often characterised by bacteremia, meningitis, arthralgia or erythema nodosum. Persons belonging to the HLA-B 27 group are prone to develop reactive arthritis.

For **diagnosis**, a sample of feces from diarrhea patients is cultured on standard (MacConkey agar) or selective agar (often with **cold enrichment**). Gram-negative rods, which are motile at 25°C and non-motile at 37°C, growing on MacConkey agar as lactose-fermenting pink colonies are

provisionally identified as *Y. enterocolitica*. Optimum temperature is 25°C. **Cold enrichment** (incubating at 4°C) for one week increases the rate of isolation.

The infection is associated with two autoimmune disorders, reactive arthritis and Reiter's syndrome. *Salmonella* and *Shigella* have also been incriminated in these syndromes.

Treatment

Gastroenteritis and mesenteric lymphadenitis do not require treatment. In the case of bacteremia, ciprofloxacin or trimethoprim-sulphamethoxazole may be given. Prevention is by maintaining food hygiene.

TRIBE EDWARDSIELLAE

EDWARDSIELLA TARDA

Edwardsiella tarda has the general properties of Enterobacteriaceae. It is a motile rod with weak fermentative ability. The name *tarda* refers to its tardy or weak fermentation of carbohydrates. Its pathogenic role is uncertain. *E. tarda* has been isolated from wounds, urine, blood and from CSF in cases of fatal meningitis.

TRIBE CITROBACTERIACEAE

CITROBACTER DIVERSUS AND CITROBACTER FREUNDII

Citrobacter is a normal intestinal inhabitant. It may cause infections of the urinary tract, gallbladder, middle ear and meninges.

These are motile bacilli which utilise citrate, produce H_2S and may or may not be lactose fermenters. Three species are recognised: i) *C. freundii* (with typical reactions), ii) *C. koseri* (formerly *C. diversus*) and
iii) *C. amalonaticus,* which does not form H_2S. **They exhibit extensive antigenic sharing with salmonellae**. Some strains (e.g., the Bhatnagar strain) have a Vi antigen serologically identical to the antigen of *S.* Typhi and *S.* Paratyphi C.

TRIBE KLEBSIELLAE

KLEBSIELLA PNEUMONIAE

Clinical Case 4

An adult male was admitted to the ICU following a road traffic accident. He was put on a ventilator due to severe respiratory distress. On the third day, his condition worsened with symptoms of high fever and features of pneumonia seen on chest X-ray. Blood culture yielded lactose-fermenting mucoid colonies, identified as Klebsiella pneumoniae. It was multidrug-resistant, but sensitive only to imipenem and piperacillin-tazobactam. He was treated with imipenem. However, he succumbed to the infection on day 10 despite aggressive management.

Morphology

This genus consists of non-motile rods, which are capsulated, short, plump and straight, measuring about 1–2 µm × 0.5–1.8 µm in size. The mucopolysaccharide capsule is often prominent and can be made out even in Gram-stained smears as halos around the rods. Klebsiellae are widely distributed in nature, occurring both as commensals in the intestines and as saprophytes in soil and water.

Classification

They have been classified into three species—*K. pneumoniae, K. ozaenae* and *K. rhinoscleromatis*—based on biochemical reactions and into over 80 serotypes based on the K antigens.

Pathogenicity

Currently, *Klebsiella* is an important cause of **health care-associated infections**. Common infections associated with *K. pneumoniae* are:

i) Pneumonia, community-acquired (CAP), in diabetics and chronic obstructive pulmonary disease (COPD) and hospital-acquired (HAP), often, ventilator-associated pneumonia (VAP)
ii) Urinary infection (including catheter-associated UTI (CAUTI hospital-acquired)
iii) Septicemia
iv) Several pyogenic infections

Pneumonia due to *Klebsiella* has high case fatality (*Clinical case* 4).

It occurs in middle-aged or older persons with risk factors such as alcoholism, chronic bronchopulmonary disease or diabetes mellitus. It can be community-acquired or hospital-acquired.

The disease is characterised by a massive mucoid inflammatory exudate of lobar or lobular distribution, involving one or more lobes of the lung.

Necrosis and abscess formation are more frequent than in pneumococcal pneumonia.

Serotypes 1, 2 and 3 are usually responsible for pneumonia. Positive blood cultures can be obtained in about 25 per cent of the cases. In recent years, a new hypervirulent hypermucoviscous variant of Klebsiella has emerged. This is associated with community-acquired infections including liver abscess, pneumonia, meningitis and endophthalmitis and the ability to metastatically spread.

K. ozaenae is a bacillus associated with ozena, a disease characterised by foul-smelling nasal discharge.

K. rhinoscleromatis causes rhinoscleroma, a chronic granulomatous hypertrophy of the nose prevalent in southeastern Europe, India and in Central America. The species *K. oxytoca* may rarely be isolated from clinical specimens.

Laboratory Diagnosis

Diagnosis is made by culturing appropriate specimens and identifying the isolate by biochemical reactions.

1. **Cultural characteristics** Klebsiellae grow well on ordinary media, forming large, dome-shaped, mucoid colonies with varying degrees of stickiness. On MacConkey agar, they grow as lactose-forming colonies. Fresh clinical isolates are often highly mucoid, almost flowing down the surface of the medium (Fig. 30.8).

Fig. 30.8 Mucoid lactose-fermenting colonies of *Klebsiella pneumoniae* (*Source:*

2. **Biochemical reactions** Klebsiellae ferment carbohydrates (glucose, lactose, sucrose and mannitol) with the production of acid and abundant gas. The IMViC reaction is – –++. Biochemical variants are common. The species can be differentiated by MR-VP, malonate and lysine. However, clinically, it is of less consequence as *K. pneumoniae* is the predominant type associated with human infection (Table 30.6).

Table 30.6 Differentiation of *Klebsiella* species

	K. pneumoniae	***K. ozaenae***	***K. rhinoscleromatis***
Gas from glucose	+	d	–
Acid from lactose	+	d	–
MR	–	+	+
VP	+	–	–
Citrate	+	d	–
Urease	+	d	–
Malonate	+	–	+
Lysine	+	d	–

Antibiotic sensitivity must be done. Many strains carry plasmids that determine multiple drug resistance by producing carbapenamases and metallo-beta-lactamases (***kpc, ndm*-1, etc.**). Recently, **hypervirulent** and **hypermucoviscous** (hvKp) strains of *Klebsiella* have appeared, which cause community-acquired infections in healthy individuals. These bacilli are carried in the gut and easily spread in the community and in hospitals, causing hospital-acquired infections. The K1 and K2 serotypes of hvKp are resistant to antibiotics.

Treatment

For bloodstream infections, 4.5 g of piperacillin + tazobactam every six hours or 3 g of cefoperazone-sulbactam every 12 hours with an aminoglycoside, e.g., amikacin 20 mg/kg/day must be given.

Recently, several strains have shown extensive drug resistance, including resistance to carbapenems like imipenem and meropenem. These are known as XDR strains. The only antibiotic that can be given in this case is colistin or polymixin (liposomal). These are very toxic and expensive. Hence care must be taken to use antibiotics judiciously.

ENTEROBACTER CLOACAE

These are normally found in feces, sewage, soil and water and rarely, in urine, pus and other pathological materials. They may be responsible for health care-associated infections. These have recently been shown to be resistant to several classes of antibiotics, making treatment difficult.

HAFNIA ALVEI

This is found in human and animal feces, sewage, soil and water, and is rarely isolated from clinical specimens.

SERRATIA MARCESCENS

Serratia forms a characteristic pink, red or magenta, non-diffusible pigment called **prodigiosin** (Fig. 30.9), which is formed optimally at room temperature. Only one species is of medical importance—*S. marcescens*. The frequency of reports of **health care-associated infections due to *S. marcescens*** is increasing. They contaminate IV fluids, surgical instruments and antiseptic solutions. The bacillus has been associated with meningitis, endocarditis, septicemia, peritonitis, respiratory infection and many other conditions. **Multiple drug resistance is common in hospital strains.**

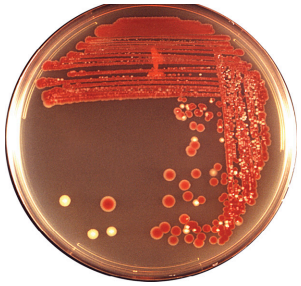


Fig. 30.9 Non-diffusible, red pigment-producing *Serratia* spp. on blood agar (*Source:* Public Health Image Library, ID 10544 / Dr. Negut / CDC)

Pantoea agglomerans, earlier known as *Enterobacter agglomerans,* is a ubiquitous bacteria found on plants, in water and in soil. It may be isolated from human and animal feces. This organism is rarely found in clinical samples.

TRIBE PROTEEAE

The tribe Proteeae are normal intestinal commensals and opportunistic pathogens like the other coliforms. The name *Proteus* refers to their pleomorphism, after the Greek god Proteus who could assume any shape. They swarm on nutrient and enriched culture plates (Fig. 30.10).

Fig. 30.10 Swarming of *Proteus* on blood agar (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Proteeae contains three genera: (i) *Proteus*, (ii) *Morganella* and (iii) *Providencia*. Most of them, except for some *Providencia* strains, produce a powerful enzyme, urease, which rapidly hydrolyses urea to ammonia and carbon dioxide.

Phenyl pyruvic acid reaction A characteristic feature which distinguishes Proteeae from other coliforms is the presence of the enzyme phenylalanine deaminase, which converts phenylalanine to phenyl pyruvic acid (PPA reaction); it is seen in all members of the tribe.

PROTEUS

The genus ***Proteus*** contains two medically important species—*P. mirabilis*, which is an important urinary and nosocomial pathogen, and *P. vulgaris,* which is found much less commonly in human infections. A characteristic putrid odour described as **'fishy' or 'seminal' odour** is produced in cultures.

All species of this genus are gram-negative, non-capsulated, pleomorphic, motile rods. They ferment several carbohydrates. *P. vulgaris* and *P. mirabilis* produce abundant hydrogen sulphide.

Swarming

P. mirabilis and *P. vulgaris* swarm on solid culture media due to the presence of flagella. Several methods have been used to inhibit swarming:

increasing the concentration of agar to 6% or incorporating chloral hydrate (1:500), sodium azide (1:500), alcohol (5–6%), sulphonamide and using surface-active agents or boric acid (1:1,000) in the medium. Swarming does not occur on MacConkey medium due to the presence of bile. Smooth, colourless colonies are formed on this medium.

Proteus species have been used as heterophile antigens to detect IgG antibodies to scrub typhus.

Weil–Felix reaction These bacilli possess somatic O and flagellar H antigens, which form the basis of Weil–Felix reaction for the diagnosis of a few rickettsial infections. This is used as a diagnostic test due to cross-reaction of these antigens (**heterophile antigens**) with some Rickettsiae. However, this test has been replaced by ELISA due to it low specificity. Three non-motile *Proteus* strains—OX2, OX19 and OXK—are used in the agglutination test.

Treatment

Proteus species are inherently resistant to many of the common antibiotics. An exception is *Pr. mirabilis* which is sensitive to ampicillin and cephalosporins.

MORGANELLA

The genus ***Morganella*** does not swarm in culture**.** The organism is rarely associated with food poisoning due to improperly cooked fish, known as scombroid food poisoning. It also causes nosocomial wound infections.

PROVIDENCIA

The genus ***Providencia*** contains three species which are associated with infections:

- *P. alcalifaciens* is sometimes seen in normal human feces but far more frequently in **diarrheal stools,** though its role in the disease is uncertain.
- *P. stuartii* is a common cause of **urinary infection** and infection in **burns**.

- *P. rettgeri* is part of the normal fecal flora of reptiles and amphibians, and sometimes causes **nosocomial infections** of the urinary tract, wounds, burns and blood.

Resistance to Disinfectants and Antiseptics

Providencia, particularly *P. stuartii,* are resistant to disinfectants such as chlorhexidine, cetrimide, benzalkonium chloride and heavy metal compounds such as silver sulphonamide. This is especially a problem in burns units, where it is a major cause of post-burn infection. It is sensitive to phenol and glutaraldehyde.

Treatment

Amikacin and ciprofloxacin are generally effective in the treatment of infections due to *Providencia*.

TRIBE ERWINIEAE

ERWINIA HERBICOLA

This organism has occasionally been isolated from respiratory and urinary tract infections in immunocompromised or hospitalised patients. They are usually found in soil and cause plant infections.

These are anaerogenic bacilli that form a yellowish pigment.

Key points

- *Escherichia coli* is an aerobe and a facultatively anaerobic, motile, gram-negative rod found as a normal commensal of the intestine. It is oxidase-negative and catalase-positive. The organisms form pink, lactose-fermenting colonies on MacConkey agar.
- *E. coli* causes diarrhea, urinary tract infection (UTI), meningitis and respiratory tract infection.
- Virulence factors include the 'P' pili in UTI; K1 capsule (neonatal meningitis); heat-labile and/or stable toxins (LT and ST) in enterotoxigenic *E. coli* (ETEC) gastrointestinal disease; adherence factor and enterocyte effacement factor in enteropathogenic *E. coli* (EPEC) disease; invasiveness in enteroinvasive *E. coli* (EIEC) and Shiga-like toxin in enterohemorrhagic *E. coli* (EHEC).

- Members of the genus *Klebsiella* are facultatively anaerobic, non-motile, gram-negative bacilli associated with multidrug-resistant nosocomial infections, urinary tract infections, septicemia, chest infections and meningitis.
- Other lactose-fermenting members of Enterobacteriaceae are *Enterobacter*, *Edwardsiella*, *Citrobacter* and *Serratia*. They may cause opportunistic infections in debilitated or immunocompromised patients and are difficult to treat as they are resistant to a wide range of antimicrobial substances.
- Members of the genus *Proteus* are non-lactose fermenting, facultatively anaerobic, motile, gram-negative bacilli that exhibit swarming on nutrient and blood agar. They are commensals of the intestine and cause urinary tract and wound infections.
- *Shigella* is a genus of the family Enterobacteriaceae; organisms of this genus are non-motile and facultatively anaerobic.
- The species can be distinguished biochemically and on the basis of serotypes. The four main species are: i) *Shigella dysenteriae*, ii) *Shigella flexneri*, iii) *Shigella sonnei* and iv) *Shigella boydii*.
- These bacteria are highly infectious, as 10–100 organisms can initiate disease. Humans are the only reservoir.
- The *Shigella* species cause dysentery by binding to M cells and invading the lamina propria, causing cell death and inflammation, leaving behind transverse superficial ulcers.
- The genus *Salmonella* is a motile bacillus that is catalase-positive and oxidase-negative.
- Two species of *Salmonella* are recognised:
    - *S. bongori* (not relevant for human infections)
    - *S. enterica, which has* 5 subspecies: includes human pathogens *S*. Typhi, Paratyphi, etc.
- The serovars that cause typhoid fever include *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Paratyphi A and *Salmonella enterica* serovar Paratyphi B.
- Laboratory diagnosis is by blood culture.
- *Salmonella* are identified by biochemical characteristics and agglutination using specific antisera against, O, H and Vi (capsular polysaccharide) antigens.
- Some patients become carriers, harbouring the bacteria in their tissues without symptoms and passing organisms in their feces for years.
- Enteric fever can be prevented by proper personal hygiene, consumption of safe drinking water and vaccines. Antibiotics are needed to treat sick individuals and to eliminate the carrier state. There is currently a live oral vaccine available for immunisation (Ty2 1a).
- The Salmonella species responsible for food poisoning are transmitted by the consumption of contaminated eggs and meat from poultry.
- There are many serovars implicated in food poisoning, the prominent ones being *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis.
- Antibiotic resistance in *Salmonella* is an emerging problem.

Questions

Essays

1. Enumerate the bacteria causing UTI and describe the laboratory diagnosis of UTI.
2. Enumerate the organisms causing diarrhea. Describe the pathogenesis and diagnosis of diarrheagenic *E. coli*.
3. Describe the pathogenesis and laboratory diagnosis of bacillary dysentery.
4. Describe the pathogenesis, laboratory diagnosis and prevention of typhoid fever.

Short Answers

1. Virulence factors of *E. coli*
2. Bacillary dysentery
3. Antibiotic resistance in *S.* Typhi
4. Detection of typhoid carriers
5. Infections by non-typhoidal *Salmonella*

Part III: Bacteriology

Chapter 31

Vibrionaceae

Chapter navigation

- *Vibrio cholerae*
- Classification
- Cholera toxins
- Pathogenesis
- Epidemiology
- Prevention and control
- Halophilic vibrios
- *Aeromonas*
- *Plesiomonas*

Vibrios are gram-negative, curved bacilli that are actively motile by means of a polar flagellum. The name 'vibrio' is derived from its characteristic vibratory motility (from *vibrare,* meaning 'to vibrate'). Vibrios are present in marine environments and surface waters worldwide. The most important member of the genus, *Vibrio cholerae,* is the causative agent of cholera. Filippo Pacini first identified, described and named the organism as *Vibrio cholerae* after isolating them from cholera patients in Florence, Italy, in 1854. Later, in 1883, Robert Koch isolated the organism from cholera patients in Egypt. Cholera went on to kill millions across the Indian subcontinent and caused pandemics across the globe. In the 1950s
Dr S. N. De described the pathogenesis of cholera and also its management by rehydration. Sadly, despite being nominated several times, Dr De did not win the Nobel Prize for his path-breaking findings.

VIBRIO CHOLERAE

Clinical Case

A 12-year-old boy from an overcrowded colony presented to the pediatric emergency department during the monsoons with complaints of profuse and frequent (10–15 times) watery diarrhea from the previous

day, accompanied by 2–3 episodes of vomiting. There was no history of fever, abdominal pain or passage of blood in stools. He complained of decreased urine output, intense thirst and leg cramps. On examination, he was found to be moderately dehydrated. He mentioned that some of the other residents of the slum were also suffering from a similar condition. The stool on gross examination had a characteristic 'rice water' appearance. The stool culture was positive for Vibrio cholerae. Management included rehydration and a single dose of azithromycin 20 mg/kg of body weight.

Morphology

The cholera vibrio is a short, curved, rod, about 1.5 × 0.2–0.4 μm in size. The cell is comma-shaped (Fig. 31.1a). Pleomorphism is frequent in old cultures. In stained films of mucus flakes from acute cholera cases, the vibrios are seen arranged in parallel rows, described as '**fish in a stream**' appearance. They are actively motile **(darting motility)**, with a single sheathed polar flagellum (Fig. 31.1b). Darting type motility can be seen microscopically in acute cholera stool or a young culture resembling a '**swarm of gnats'**. *Vibrio cholerae* are gram-negative and non-acid-fast.

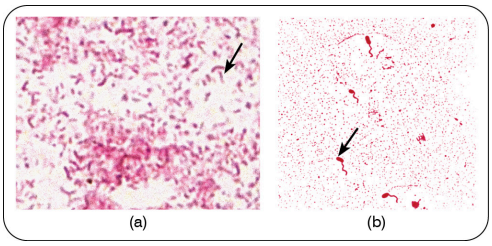


Fig. 31.1 *Vibrio cholerae;* (a) comma-shaped gram-negative rods and (b) digitally-enhanced photomicrograph of *Vibrio cholerae* stained by Leifson flagella stain under 320× magnification showing polar flagella (*Source* (a) Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry and (b) Public Health Image Library, ID 1034 / Dr. William A. Clark / CDC)

Classification

Precise criteria have been laid down for differentiating *Vibrio* from related genera *Aeromonas* and *Plesiomonas* (Table 31.1).

Table 31.1 Differentiation of *Vibrio, Aeromonas* and *Plesiomonas*

Genus	Oxidation–Fermentation				
	(Hugh–Leifson test)		Utilisation of amino acids		
	Oxi-dation	Ferme-ntation	Lysine	Argi-nine	Orni-thine
Vibrio	+	+1	+	–	+

Aeromonas	+	+2	–	+	–
Pseudomonas	+	–	V	V	V
Plesiomonas	+	+	+	+	+

1 = no gas produced; 2 = gas may or may not be produced; V = reaction variable

1. Based on the requirement of sodium chloride, they are divided into **halophilic** and **non-halophilic** vibrios.

   - Halophilic: *V. parahemolyticus*, *V. alginolyticus*, *V. vulnificus*
   - Non-halophilic: *Vibrio cholerae*

2. Based on the fermentation of mannose, sucrose and arabinose, **Heiberg** classified vibrios into six groups (Table 31.2). Two more groups were added later. Cholera vibrios belong to Group I.

Table 31.2 Heiberg grouping of vibrios

Groups	Fermentation of		
	Mannose	Sucrose	Arabinose
I	+	+	–
II	–	+	–
III	+	+	+
IV	–	+	+
V	+	–	–
VI	–	–	–
VII	+	–	+
VIII	–	–	+

3. A **serological classification** was introduced by **Gardner** and **Venkatraman** (1935) (Fig. 31.2). Based on the major somatic (O) antigen, Group A vibrios were further classified into 'subgroups' (now called O serogroups or serovars), 139 of which are currently known. All isolates from epidemic cholera (until 1992) belonged to serogroup O-1 (referred to as **agglutinable vibrios**). Other vibrio

isolates which did not agglutinate with the O-1 antiserum came to be known as **non-agglutinable vibrios**.

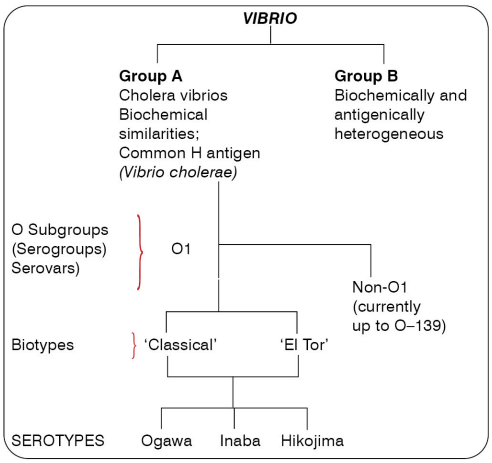


Fig. 31.2 Gardner and Venkatraman's classification (updated)

Biotypes

In Gardner and Venkatraman's classification, all isolates from epidemic cholera were said to belong to Group O-1, called **classical *V. cholerae.***

> Other vibrios isolated from Haj pilgrims at the Tor quarantine station on the Sinai Peninsula came to be called the **El Tor vibrios**. They were identical to cholera vibrios in all laboratory tests except that they lysed sheep erythrocytes and gave a positive Voges–Proskauer reaction. In 1937, El Tor vibrios were recognised as endemic in Celebes (Sulawesi) and Indonesia and caused an epidemic choleraic disease.

Table 31.3 lists the differentiating characteristics of El Tor and classical Vibrio which are applicable in the laboratory even today.

Table 31.3 Differentiation between classical cholera and El Tor vibrios *(biotypes)*

Test	Classical cholera	El Tor
Hemolysis	–	+
Voges–Proskauer	–	+
Chick erythrocyte agglutination	–	+
Polymyxin B sensitivity	+	–
Group IV phage susceptibility	+	–

El Tor phage 5 susceptibility	–	+

Serotypes

Based on minor surface antigenic characteristics, classical and El Tor biotypes of cholera vibrios were classified as **Ogawa and Inaba**. A third serotype, Hikojima, is agglutinated by both Ogawa and Inaba antisera (Table 31.4). The difference is only of epidemiological significance.

The non-O-1 vibrios (the so-called **non-agglutinating vibrios**) have been classified currently up to 139 serogroups. O-139, identified in 1992, causes, epidemics of cholera.

Table 31.4 O serotypes of cholera vibrios

Serotype	O antigens
Ogawa	AB
Inaba	AC
Hikojima	ABC

Phage typing Phage typing schemes have been standardised for classical and El Tor biotypes as well as for O-139 vibrios.

Molecular typing New molecular methods like ribotyping have added further refinements to strain typing.

Resistance

Cholera vibrios are susceptible to heat, drying and acids but resist high alkalinity. They are destroyed at 55°C in 15 minutes. Survival in water is influenced by its pH, temperature, salinity, presence of organic pollution and other factors.

- **El Tor vibrio survives longer than the classical cholera vibrio.**
- **In grossly contaminated waters, such as those of the river Ganga, the vibrios do not survive for any length of time due to the large amounts of vibriophages present**.
- In clean tap water, they survive for thirty days.
- In untreated soil, they may survive for several days.

- On fruits, they survive for 1–5 days at room temperature and for a week in the refrigerator.
- Vibrios are susceptible to common disinfectants.
- They are killed in a few minutes in the gastric juice of normal acidity but may survive for 24 hours in achlorhydric gastric juice.

Cholera

Cholera is an acute diarrheal disease caused by *V. cholerae*. Natural infection with cholera occurs only in humans. Infection is through the oral route. Its source is contaminated water or food. In its most severe form, it presents as profuse, painless, watery diarrhea and copious effortless vomiting; this may lead to hypovolemic shock and death in less than 24 hours. With fluid replacement and antibiotics, the disease may last 4–6 days (the patient may pass a total volume of liquid stool equal to twice his body weight).

All the clinical features of severe cholera result from this massive loss of fluid and electrolytes.

Rice water stool

The cholera stool is typically a colourless watery fluid with flecks of mucus (*Clinical case*), said to resemble water in which rice has been washed (hence called '**rice water stools**'). It has a characteristic inoffensive sweetish odour. In composition, it is a bicarbonate-rich isotonic electrolyte solution, with little protein. Its outpouring leads to the diminution of extracellular fluid volume, hemoconcentration, hypokalemia, base-deficit acidosis and shock.

- **Incubation period** varies from less than 24 hours to about five days. The clinical illness may begin slowly with mild diarrhea and vomiting in 1–3 days or abruptly with sudden, massive diarrhea.
- **Incidence** of mild and asymptomatic infections is more with El Tor vibrios than with the classical cholera vibrios.
- **Complications** are muscular cramps, renal failure, pulmonary edema, cardiac arrhythmias and paralytic ileus.

- **Clinical severity** of cholera varies widely, from the rapidly fatal disease to a transient asymptomatic colonisation of the intestine by the vibrios.

Pathogenesis

Historically, a number of animal models have been developed to understand the pathogenic mechanisms in cholera. The first of these was the rabbit ileal loop model of S. N. De and Chatterjee (1953). Injection of cholera culture or culture filtrate into the **ligated ileal loop** caused fluid accumulation and ballooning.

Mechanism of action

Vibrios reach epithelial cells of the small intestine by chemotaxis, motility, mucinase and other proteolytic enzymes. A hemagglutinin-protease (formerly known as cholera lectin) cleaves mucus and fibronectin. **Adhesion to the epithelial surface and colonisation is facilitated by special fimbria—toxin co-regulated pilus (TCP).** Throughout the course of infection, the vibrios remain attached to the epithelium but do not damage or invade the cells. **The changes induced are biochemical rather than histological.**

Toxin-mediated action

Genetic control of enterotoxin

Vibrios multiplying on the intestinal epithelium produce an enterotoxin called **cholera toxin or CT (Ctx)** which is very similar to the heat-labile toxin (LT) of *E. coli,* though CT is far more potent than LT in biological activity. The most important virulence **gene** is activated by *ToxR* which encodes the production of **cholera toxin** (CT). It is also postulated that CT production is due to the presence of a phage integrated with the bacterial chromosome. It can also replicate as a plasmid, which can be transmitted to non-toxigenic strains, rendering them toxigenic. CT, TCP and other virulence factors are regulated by the *ToxR* gene product, **ToxR protein**, which is present in pathogenic strains and absent in most non-pathogenic strains. This element has been referred to as the **TCP pathogenicity island**. The *ctxAB* genes are located on the CTX genetic element; a portion of the CTX element, is now known to correspond to the genome of a filamentous bacteriophage (**designated CTX phage**). An essential CTX phage assembly

gene called ***zot*** has also been reported to encode a biological activity called zonula occludens toxin.

Mechanism of action of cholera toxin (CT)

CT is a protein of MW of approximately 87kDa and consists of 6 units; **1 A surrounded by 5 B subunits**. The **B (binding) units** attach to the GM_1 ganglioside receptors on the surface of jejunal epithelial cells. The **A (active) subunit**, is then transported into the enterocyte, whereupon it dissociates into two fragments: A_1 and A_2. The A_2 fragment only links biologically active A_1 to the B subunit. The A_1 fragment causes prolonged **activation of cellular adenylate cyclase** and the accumulation of cAMP. This leads to the outpouring of intracellular electrolytes Na, Cl and K and large quantities of water (Fig. 31.3) into the small intestinal lumen, leading to watery diarrhea. Chloride (Cl) secretion occurs through cystic fibrosis **transmembrane conductance regulators (CFTR)** which are the transport regulators in the intestine. The fluid secreted is isotonic with plasma but contains much more of potassium and bicarbonate. The toxin also inhibits intestinal absorption of sodium and chloride by the microvilli. All clinical manifestations and complications in cholera result from the massive water and electrolyte depletion.

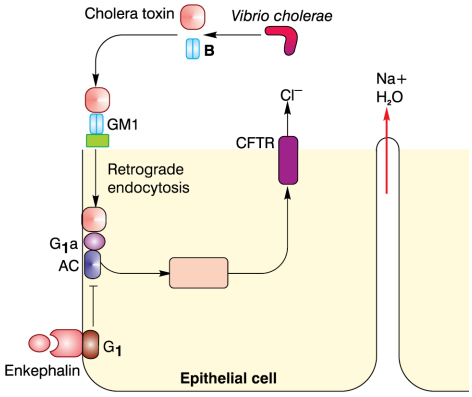


Fig. 31.3 Attachment of subunit B to GM_1 ganglioside on epithelial cells and entry of subunit A of the cholera toxin into the intestinal epithelial cells leading to hypersecretion of sodium chloride and water

In vitro activity of cholera toxin can be tested by the following methods:

- CT exhibits elongation of Chinese hamster ovary (CHO) cells in culture and histological changes in adrenal tumour (Y_1) cell

culture and Vero cells.

- It increases skin capillary permeability, also called the '**permeability factor**' (**PF**). It can be demonstrated by the **skin blueing test**—CT is injected intradermally in rabbits or guinea pigs and pontamine sky blue is injected intravenously afterwards; the site of injection of the toxin becomes blue.
- CT can also be estimated by ELISA. CT is antigenic and induces production of neutralising antitoxins.
- **CT can be toxoided.**

Cholera vibrios also possess the lipopolysaccharide O antigen (LPS and endotoxin), as in gram-negative intestinal bacilli, but plays no role in the pathogenesis of cholera. However, it is responsible for the immunity induced by killed vaccines.

Epidemiology

Cholera can occur in many forms: sporadic, endemic, epidemic or pandemic. The large deltaic area of the rivers Ganga and Brahmaputra in Bengal is its homeland, where it has been known to exist since ancient times.

Historical perspective

From 1817 to 1923, cholera caused by the **classical biotype** spread from Bengal in six separate pandemic waves involving most parts of the world. The seventh pandemic originated in Sulawesi (Celebes), Indonesia, in 1961, caused by the **El Tor biotype,** which replaced the classical biotype. After spreading to Hong Kong and the Philippines, it spread steadily westwards, invading India in 1964. In January 1991, the pandemic reached Peru, thus encircling the globe in thirty years. The seventh pandemic was different from the others. It was the first to have originated from outside the Indian subcontinent. It was also the first to have been caused by the El Tor biotype.

Epidemiology of El Tor

The severity of illness is much less than that of classical cholera, with a large proportion of mild and asymptomatic infections. Mortality is low and the carrier rate high. El Tor vibrios tend to remain endemic in many new geographic areas, causing periodic epidemics. The El Tor vibrio has proved to be much hardier than the classical vibrios, capable of surviving in the environment for much longer. In India, the classical vibrio has been replaced by El Tor; however, in Bangladesh, the classical vibrio has staged

a comeback.

An event of great significance was the sudden emergence of non-O-1 *V. cholerae* as the cause of epidemic cholera (eighth pandemic). In **October 1992, a new non-O-1 vibrio was isolated from a cholera outbreak in Madras (Chennai).** Similar outbreaks soon followed in different parts of India. By January 1993, the new strain had become epidemic in Bangladesh as well. In the affected areas, this strain replaced the El Tor vibrios as the epidemic and environmental serovar. It also showed a tendency to be more invasive, causing bacteremic illness in some.

The new epidemic strain was designated **serovar O-139** (or **O-139 Bengal**). Unlike the O-1 cholera vibrio, **the O-139 vibrio is capsulated.** As it possessed novel surface antigens, the O-1 strain vaccines could not protect against O-139 infection. There was no natural antibody against the strain in any human population then. It was, therefore, considered likely that the O-139 strain would initiate the next pandemic of cholera. The new strain continued spreading eastwards to the Southeast Asian countries and westwards to Pakistan, China and some parts of Europe. But surprisingly, by 1994, the El Tor strain regained its dominance and the threat of an O-139 pandemic diminished. **Both O-1 El Tor and O-139 had begun to co-exist in endemic areas but are now declining.**

Ten months after Haiti's catastrophic earthquake in 2010, a cholera outbreak began to spread through the country. Large-scale movement of persons, as occurs during fairs and festivals, has traditionally been associated with the spread of cholera.

The persistence of the vibrio during the inter-epidemic periods may be maintained by continuous transmission of subclinical or mild infection. It is now known that the natural habitat of cholera vibrios is the saline waters of coastal seas and brackish estuaries, where they can persist for long periods, particularly in association with small crustaceans such as copepods, crabs or plankton. Cycles of transmission are perpetuated when bacteria are shed into water sources by fecal contamination.

A significant difference in susceptibility to cholera has been reported in relation to blood groups, group O persons being the most susceptible and group AB the least. The reason for this is not known.

Laboratory Diagnosis

Diagnosis is based on isolation and identification of *V. cholerae* from stool or rectal swabs.

1. **Specimen** **Stool**, collected in the acute stage of the disease, before the administration of antibiotics, is the most useful specimen. **Rectal swabs** made of good quality cotton wool, may be used to absorb about 0.1–0.2 mL of watery fluid from the diarrheic stool.

Collecting samples from the pan is not recommended**.** Vomitus is not useful.

Transport As cholera vibrios may die in a few hours in tropical temperatures, it is necessary to preserve the specimen at 4°C or in some appropriate holding medium. Stool samples may be preserved in VR fluid or Cary–Blair medium for long periods.

2. **Microscopy** **Diagnosis by direct microscopic examination of cholera stool is not recommended as the results are not reliable**.

For **rapid diagnosis**, the characteristic **darting motility of the vibrio and its inhibition by specific antiserum** can be demonstrated under the dark field or phase-contrast microscope, reliably after enrichment for six hours.

Demonstration of vibrios in stools by direct immunofluorescence is not useful.

3. **Cultural characteristics** *Vibrio cholerae* grows aerobically at a temperature range of 16–40°C (optimum 37°C). **Growth is better in an alkaline medium**, the range of pH being 6.4–9.6 (optimum 8.2). NaCl (0.5–1%) is required for optimal growth, though high concentrations (6% and above) are inhibitory. They are salt-tolerant (unlike *Vibrio parahaemolyticus* which is halophilic and needs a higher concentration of NaCl). The **enrichment media** should be incubated for 6–8 hours including transit time. The specimens sent in holding media should be inoculated into enrichment media and incubated for 6–8 hours before being streaked on a selective and a non-selective medium.

The **media** used vary in different laboratories. Commonly used media are, MacConkey agar as non-selective and TCBS agar as selective plates. Generally, the plates are examined after overnight incubation at 37°C.

- On **nutrient agar**, after overnight growth, colonies are moist, translucent, round discs, about 1–2 mm in diameter, with a

bluish tinge in transmitted light. The growth has a distinctive odour.

- On **MacConkey agar**, the colonies are colourless at first but become pink on prolonged incubation due to the late fermentation of lactose.
- On **blood agar**, colonies are initially surrounded by a zone of greening, which later becomes clear due to hemodigestion.
- In **gelatin stab** culture, funnel-shaped or napiform (turnip-shaped) liquefaction occurs in three days at 22°C.
- In **peptone water**, growth occurs in about six hours as a fine surface pellicle.
- A number of **special media** have been employed for the cultivation of cholera vibrios.

  i) ***Holding or transport media***

  - **Venkatraman–Ramakrishnan (VR)** medium has a pH to 8.6–8.8. It consists of sea salt (20 g) and peptone (5 g) in distilled water. In this medium, vibrios do not multiply but remain viable for several weeks.
  - The **Cary–Blair medium** has a pH 8.4 with buffered solution of sodium chloride, sodium thioglycolate, disodium phosphate and calcium chloride. It is also a suitable transport medium for *Salmonella* and *Shigella* as well as for vibrios.
  - **Autoclaved seawater** also serves as a holding medium.

  ii) ***Enrichment media*** (can also be used as transport media)

  - Alkaline peptone water (APW) has a pH of 8.6.
  - Monsur's taurocholate tellurite peptone water has a pH of 9.2.

  iii) ***Media for culture and isolation***

  - **Thiosulphate citrate bile salt sucrose (TCBS)** medium, containing thiosulphate, citrate, bile salts and sucrose, is very widely used. Cholera vibrios, due to the fermentation of sucrose, produce large, yellow, convex colonies (Fig. 31.4), which may become green on continued incubation.

Fig. 31.4 Yellow colonies on TCBS (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- **Alkaline bile salt agar (BSA)** at pH 8.2 is a simple medium that has stood the test of time and is still widely used. On this medium, colonies are similar to those on nutrient agar.
- In Monsur's gelatin taurocholate trypticase tellurite agar **(GTTA)**, cholera vibrios produce small, translucent colonies with a greyish-black centre and a turbid halo. The colonies become 3–4 mm in size in 48 hours.

4. **Identification** Vibrio colonies may be identified by the 'string test'. A loopful of the growth is mixed with a drop of 0.5% sodium deoxycholate in saline on a slide. The suspension becomes mucoid and forms a 'string' when the loop is drawn slowly away from the suspension (Fig. 31.5). The string is formed by DNA material that is released from the vibrios as a result of the lysis of the cells by sodium deoxycholate.

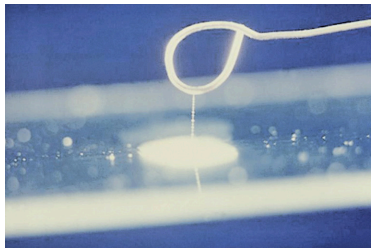


Fig. 31.5 String test used to differentiate *Vibrio* spp. from *Aeromonas* spp. and *Plesiomonas*

5. **Biochemical reactions** The isolate is subjected to studies including the oxidase test, utilisation of amino acids, lysine, arginine and ornithine, fermentation of sugars including sucrose, mannose and arabin ose, hemolysis, VP, polymyxin B sensitivity and susceptibility to cholera phage

IV.

Vibrios produce the enzyme oxidase; carbohydrate metabolism is fermentative, producing acid, but no gas. Cholera vibrios ferment glucose, mannitol, maltose, mannose and sucrose. Lactose may be split very slowly. Indole is formed and nitrate is reduced to nitrite. These two properties contribute to the **cholera red reaction**.

Cholera red reaction (nitroso-indole reaction) is tested by adding a few drops of concentrated sulphuric acid to a 24-hour peptone water culture (Fig. 31.6). With cholera vibrios, a reddish-pink colour develops due to the formation of nitroso-indole.

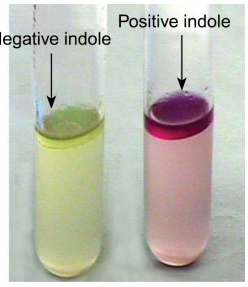


Fig. 31.6 Cholera red reaction

Vibrios elaborate several enzymes including collagenase, elastase, chitinase, nucleotidase, decarboxylase, lipase, mucinase and **neuraminidase** (**receptor-destroying enzyme**).

Confirmation by serogrouping Colonies suggestive of vibrios should be tested by agglutination with cholera O subgroup I serum (cholera non-differential serum). If positive, further typing with specific Ogawa and Inaba sera must be done for typing. If agglutination is negative with one colony, it is essential to repeat the test with at least five more colonies. The isolate is further tested for differentiation between El Tor and classical cholera vibrios (Table 31.3).

Isolates of vibrios that are not agglutinated by the O subgroup I serum should not be ignored because non-O-1 vibrios are known to produce cholera-like disease. Specific antiserum against O-139 is available.

The national reference laboratory for identification (including phage typing) and repository is the International Reference Centre for vibrio at the National Institute of Cholera and Enteric Disease (NICED) at Kolkata.

Testing of water samples Two methods are employed.

i) **Enrichment method** In this method, 900 mL of water is added to 100 mL tenfold concentrated peptone water at pH 9.2, incubated at 37°C for 6–8 hours and a second enrichment done before plating on selective media.

ii) **Filtration technique** In this method, the water to be tested is filtered through a Millipore membrane. The membrane is then placed directly on the surface of a selective medium and incubated. Colonies appear after overnight incubation. Sewage should be diluted in saline, filtered through gauze and treated as was described for water.

Immunity

Immunity may be local, in the intestine, or systemic. In cholera, the vibrios remain confined to the intestine, where they multiply and elaborate the enterotoxin which is responsible for the disease. Immunity, therefore, is directed against the bacterium or against the toxin—antibacterial or antitoxic. Natural infection confers some amount of immunity but it does not last for more than 6–12 months and re-infections after this period are known to occur.

Local immunity is conferred by copro antibodies which consist of IgG, IgM and IgA which are local antibodies in the intestine, and they appear in the feces (hence known as copro antibodies). Their protective role is short-lived.

Immunisation with killed vaccines induces only short-term antibacterial immunity. The protective effect of these vaccines, especially purified somatic antigens used as vaccines, is short-lived, and appears to be serotype-specific but not biotype-specific.

Vaccines

Parenteral vaccines

The vaccines used traditionally are **killed suspensions containing 8,000 million *V. cholerae* per mL**, composed of equal numbers of Ogawa and Inaba serotypes. These are given by subcutaneous or intramuscular injection. Many laboratories employ classical cholera and El Tor vibrios in equal numbers in the vaccine.

Strain O-139 vaccine has also been prepared.

Several controlled field trials in endemic areas have shown that the protection afforded by the vaccine does not exceed 50–60 per cent; the duration of protection is only 3–6 months. Also, injectable vaccines do not provide any local immunity in the intestinal mucosa. They are also unacceptably reactogenic.

Oral vaccines

Two types of oral vaccines are under trial:

1. **Killed oral whole cell vaccines** with and without the inclusion of the B subunit of CT
2. **Live oral vaccines** with classical, El Tor and O-139 strains, with their toxin genes deleted

Oral inactivated, or non-live cholera vaccines are available. These cholera vaccines are World Health Organization (WHO) prequalified but not widely used. As per the WHO release, "more than 20 million doses of oral cholera vaccines (OCVs) have been used in mass vaccination campaigns. The campaigns have been implemented in:

- Areas experiencing an outbreak
- Areas at heightened vulnerability during humanitarian crises
- Among populations living in highly endemic areas, known as **'hotspots'"**

Two doses of the vaccines are required for full protection (minimum 1 week and maximum 6 weeks apart). Three doses are required for children aged 2–5 years (minimum 1 week and maximum 6 weeks apart).

Treatment

Cholera can be treated easily. The mainstay of treatment consists essentially of prompt and adequate replacement of fluids and electrolytes by oral rehydration solution (ORS). This is done, either alone or supplemented by intravenous fluids. Cereal-based preparations are equally effective and are usually more acceptable**. Antibacterial therapy is of secondary importance**. Initially, cholera vibrios were uniformly susceptible to all antibiotics active against gram-negative bacilli but since 1979, multiple drug-resistant strains have become increasingly common. Doxycycline is recommended as first-line treatment for adults, while azithromycin is recommended as first-line treatment for children and pregnant women.

Prevention

The prevention of cholera is by general measures such as provision of protected water supply and improvement of environmental sanitation. In addition, vaccination continues to be the most widely used method of prevention in endemic areas.

VIBRIO MIMICUS

V. mimicus can be differentiated by its failure to ferment sucrose. Like *V. cholerae*, it grows best at low salt concentrations (0.5–1.0%). It has been responsible for many sporadic cases of diarrheal disease on the Gulf Coast of the USA. **Infection is acquired by eating seafood, especially oysters.** The disease is self-limiting. Clinical manifestations resemble those caused by
V. parahaemolyticus.

HALOPHILIC VIBRIOS

Vibrios that have a high requirement of sodium chloride are known as halophilic vibrios. Their natural habitat is sea water and marine life. Some halophilic vibrios have been known to cause human disease—*V. parahaemolyticus, V. alginolyticus* and *V. vulnificus.*

Vibrio parahaemolyticus

V. parahaemolyticus is an enteropathogenic, halophilic vibrio, originally isolated in 1951 in Japan as the causative agent of an outbreak of food poisoning caused by seafish. Infection is common in the coastal seas, where it is found in fish and arthropods such as shrimps and crabs, and molluscs such as oysters. In Kolkata, it has also been found in small pond fish.

It resembles the cholera vibrio, except that it is capsulated and shows bipolar staining. Unlike other vibrios, it produces peritrichous flagella when grown on solid media while polar flagella are formed in liquid cultures. **It grows only in media containing NaCl.**
V. parahaemolyticus tolerates salt concentrations up to 8% but not 10%. The optimum salt concentration is 2–4%. **On TCBS agar, its colonies are green** (as they do not ferment sucrose) with an opaque, raised centre and

flat translucent periphery (note that *V. cholerae* colonies are yellow in colour). The string test is positive.

Like *Vibrio cholera,* it is oxidase-, catalase-, nitrate-, indole- and citrate-positive. Glucose, maltose, mannitol, mannose and arabinose are fermented, producing acid only. Lactose, sucrose, salicin, xylose, adonitol, inositol and sorbitol are not fermented.

It is killed at 60°C in 15 minutes. It does not grow at 4°C but can survive refrigeration.

Three antigenic components have been recognised: somatic O, capsular K and flagellar H antigens. Serotyping is based on the O and K antigens; 12 O groups and 59 distinct K antigens have been recognised.

Not all strains of *V. parahaemolyticus* cause disease in human beings.

Strains isolated from environmental sources (such as water, fish, crabs or oysters) are nearly always non-hemolytic when grown on a special high-salt blood agar (**Wagatsuma agar**). Strains from human patients are almost always hemolytic. This is called the **Kanagawa phenomenon** and is due to a heat-stable hemolysin.

The significance of hemolysis is not known but it is used as a laboratory test for pathogenicity; only the **Kanagawa-positive strains are pathogenic.** No enterotoxin has been identified.

Pathogenesis

V. parahaemolyticus causes food poisoning associated with marine food. It also causes acute diarrhea, unassociated with food poisoning. The vibrio is believed to cause enteritis by invasion of the intestinal epithelium. Abdominal pain, diarrhea, vomiting and fever are the usual signs. Feces contains cellular exudate and often also blood. Dehydration is of moderate degree and recovery occurs in 1–3 days. Cases are more common in summer, and in adults than in children. *V. parahaemolyticus* is found in sea fish from other parts of India but human cases are much less frequent. Rarely, it may cause wound infections, and in more severe cases, sepsis.

Treatment

The infection is usually self-limiting. However, in severe cases of gastroenteritis, doxycycline is the antibiotic of choice. For uncomplicated

wound infections, minocycline or doxycycline may be used.

Vibrio alginolyticus

This halophilic vibrio resembles *V. parahaemolyticus* in many respects and was formerly considered a biotype of the same.

It has higher salt tolerance, is VP-positive and ferments sucrose (Table 31.5). It is frequently found in sea fish.

Its status as a human pathogen is uncertain. It has been **associated with infections of the eyes, ears and wounds** in human beings exposed to seawater.

Table 31.5 Some characteristics of *V. parahaemolyticus* and *V. alginolyticus*

Test	*V. parahaemolyticus*	*V. alginolyticus*
Indole	+	+
VP	–	+
Nitrate reduction	+	+
Urease	–	–
Sucrose fermentation	–	+
Swarming	–	+
Growth in 0% NaCl	–	–
7% NaCl	+	+
10% NaCl	–	+

Vibrio vulnificus

V. vulnificus, previously known as L+ vibrio, is a marine vibrio of medical importance. It is VP-negative and ferments lactose but not sucrose. It has a salt tolerance of less than 8%. It causes two types of illness:

1. Wound infection following contact of open wounds with seawater
2. In immunocompromised hosts, particularly those with liver disease, following ingestion of the vibrio, usually in oysters, it penetrates the gut mucosa but does **not** cause gastrointestinal manifestations; it enters the bloodstream from the gut and rapidly leads to **septicemia with high mortality**

AEROMONAS AND PLESIOMONAS

Besides the genus *Vibrio*, the family Vibrionaceae also contains the genera *Aeromonas* and *Plesiomonas*, some members of which have been associated with human lesions.

Aeromonas hydrophila, originally isolated from frogs, in which it causes the 'red leg disease', has been reported in many cases of **diarrhea** and from some **pyogenic** wound infections in humans. *Plesiomonas shigelloides* also has been reported from diarrheal disease. Both these are oxidase-positive, polar-flagellated, gram-negative rods and may be mistaken for vibrios. They may be differentiated from vibrios by biochemical tests such as utilisation of amino acids.

Key points

- Members of the genus *Vibrio* are gram-negative, curved bacilli which exhibit motility, are facultative anaerobes and catalase- and oxidase-positive.
- *Vibrio cholerae* is the most important cause of human disease and causes cholera. *Vibrio parahaemolyticus* and *Vibrio vulnificus* are also sometimes implicated in human infections.
- *V. cholerae* possesses O-1 or O-139 somatic antigens, and O-1 isolates are subtyped as AB (Inaba), AC (Ogama) or ABC (Hikojima) and as two biovars, classical and El Tor.
- Vibrios grow on selective culture media such as thiosulphate-citrate-bile salts-sucrose (TCBS) agar to produce yellow colonies.
- Cholera can be prevented by proper treatment of drinking water.
- Cholera is treated with rehydration therapy and, in severe cases, with tetracycline or doxycycline to shorten the course of the disease.
- *Vibrio parahaemolyticus* is halophilic (has an exceptionally high requirement for sodium chloride) and is found in prawns and other seafood. It also produces an enterotoxin but its effect is much milder than that of cholera.
- Other vibrios (*Vibrio vulnificus*) occasionally cause human disease, including traumatic wound infections and, rarely, eye infections.

Questions

Essays

1. Describe the morphology, pathogenesis and laboratory diagnosis of cholera.
2. Describe the epidemiology of cholera and its preventive strategies.

Short Answers

1. Cholera vaccines
2. Cholera toxin
3. O-139 *V. cholerae*
4. Halophilic vibrios
5. Differences between classical and El Tor vibrios

Part III: Bacteriology

Chapter 32

Gram-Negative Non-fermenters

Chapter navigation

- *Pseudomonas aeruginosa*
- Virulence factors
- Resistance
- Health care-associated infections

Gram-negative non-fermenters are a group of unrelated bacteria that have gained notoriety in the recent past due to their resistance to a wide spectrum of antibiotic drugs and due to their involvement in health care-associated infections resulting in high morbidity and mortality. They are ubiquitous in nature, often found in the environment, soil and water. They can survive adverse conditions and are often known to contaminate and survive in antiseptics and disinfectant solutions that are used in the hospitals.

They either break down carbohydrates by the oxidative pathway (**non-fermenters**: *Pseudomonas*) or do not use carbohydrate pathways for energy source (**non-utilisers**: *Flavobacterium*). These organisms are generally resistant to commonly used antibiotics in the hospital. *Pseudomonas* and *Burkholderia* are large groups of aerobic, non-sporing, gram-negative bacilli widely distributed in soil and water, while *Acinetobacter* is a gram-negative coccobacillus found in moist environments, both in the hospital and the community.

PSEUDOMONADS

Pseudomonads are hardy organisms, mostly saprophytic, which colonise the hospital environment, equipment and moist areas. They are motile with polar flagella. Some of them are pathogenic to plants, insects and reptiles. A few cause human infection, typically, health care-associated infections and infections in immunocompromised individuals.

Based on molecular analysis, pseudomonads have been re-classified and many former *Pseudomonas* species re-allocated to new genera such as *Burkholderia* and *Stenotrophomonas*.

Clinical Case 1

A 3-year-old boy presented with a history of repeated respiratory tract infections with productive cough. Further investigations showed that the sweat test was positive for high sodium chloride concentration, suggestive of cystic fibrosis. Culture of sputum showed growth of transparent, colourless colonies with a mucoid character. The organisms were oxidase-positive, motile and did not break down glucose fermentatively. These were identified as Pseudomonas aeruginosa*. Based on the antibiotic susceptibility, the boy was started on a combination of piperacillin-tazobactam and amikacin along with supportive therapy of airway clearance and nutritional supplement.*

PSEUDOMONAS AERUGINOSA

Morphology

It is a slender, gram-negative bacillus, 1.5–3 × 0.5 µm in size, actively motile with a polar flagellum. Mucoid strains, particularly isolates from cystic fibrosis patients, have an abundance of **extracellular polysaccharides composed of alginate polymers** (*Clinical case 1*). This forms a loose capsule (glycocalyx) in which microcolonies of the bacillus are enmeshed and protected from host defences.

Virulence Factors

- **Exotoxin A** has a mechanism of action similar to that of the diphtheria toxin. It also has A (active) and B (binding) subunits and inhibits protein synthesis. Good antibody response to exotoxin A is considered a favourable sign in severe infections with *P. aeruginosa*.
- Other extracellular enzymes and toxins include **proteases**, **elastases**, **hemolysins** and **enterotoxin**.
- The **slime layer** acts as a capsule in enhancing virulence. In addition, the ability to form biofilms promotes infection in hospitalised patients.

The **factors promoting infection** are:

- Breach in primary body defences
- Bacterial pili (favour adhesion)
- Bacterial exoproducts (elastase, exotoxin A and exoenzyme S)
- Lipopolysaccharide (cell wall) and the alginate glycocalyx

The **factors** contributing to its **ability to cause an infection** are:

- Pigment production that causes tissue injury
- Multidrug resistance
- Multi-disinfectant resistance
- Survival in wide temperature range

Resistance

The bacillus is killed at 55°C in one hour but **exhibits a high degree of resistance to chemical agents**. It is resistant to the common antiseptics and disinfectants such as quaternary ammonium compounds, chloroxylenol and hexachlorophane and may even grow profusely in bottles of such antiseptic lotions kept for use in hospitals. *P. aeruginosa* can grow in **Dettol** or **cetrimide selective medium**. It is sensitive to acids, beta-glutaraldehyde, silver salts and strong phenolic disinfectants. Its susceptibility to silver has been applied clinically in the use of silver sulphonamide compounds as a topical cream for burns.

Epidemiology

Pseudomonas causes a wide variety of infections (Table 32.1). The bacterium is able to establish its pathogenesis if it is introduced into devitalised tissue, through disrupted skin or mucous membrane. The process is facilitated in neutropenia along with bacterial factors.

- **Community-acquired infections by *P. aeruginosa*** are suppurative otitis and respiratory tract infections in cystic fibrosis patients.
- **Health care-associated infections by *P. aeruginosa* are observed among the following high-risk groups:**
    - **Immunocompromised patients**
    - **Diabetics**

- **Patients with cystic fibrosis**
- **Patients with malignancies** (leukemia)

The role of *P. aeruginosa* in hospital infections is due to its resistance to common antibiotics and antiseptics, and its ability to establish itself widely in hospitals. Pseudomonas survives on equipment such as respirators and endoscopes, medicines such as lotions, ointments and eye drops and even stocks of distilled water and articles such as bedpans. *P. aeruginosa* is present on the skin of the axilla and perineum in some persons.

Table 32.1 Types of localised and disseminated infections associated with *Pseudomonas*

Localised Infections	Generalised infections
Wounds and bedsores	Septicemia
Eye infections, ear infections (malignant otitis externa) and nail infections	Endocarditis
Secondary infection in burns patients	IV-line-associated bloodstream infections
Iatrogenic meningitis (following lumbar puncture)	Ventilator-associated pneumonia
Ecthyma gangrenosum	Catheter-associated urinary tract infections
Necrotising fasciitis and cellulitis	Community-acquired enteric illness, leading to sepsis and necrotising enteritis (Shanghai fever)
Chronic obstructive pulmonary lung disease (COPD)	

Laboratory Diagnosis

As *P. aeruginosa* is a frequent contaminant and coloniser in the hospital setting, isolation of the bacillus from a specimen should not always be taken as proof of its etiological role. Repeated isolation and clinical correlation help confirm the diagnosis.

1. **Cultural characteristics** It is an obligate aerobe breaking down carbohydrates by the oxidative pathway. Growth occurs at a wide range of

temperatures, 6–42°C, the optimum being 37°C.

- It grows well on **ordinary media,** producing large, translucent, irregular colonies, with a distinctive, musty or earthy smell. The colonies often produce diffusible pigment (green colour). Iridescent patches with a metallic sheen may also be seen in cultures.
- **MacConkey medium** It forms non-lactose-fermenting colonies.
- **Blood agar** Many strains are hemolytic on blood agar.
- **Broth** It forms a dense turbidity with a surface pellicle due to the abundance of oxygen on the surface.

Identification of pigmented strains of Pseudomonas from clinical specimens is easy and the reduction of nitrate to nitrogen gas helps in the identification. It may be necessary to use selective media such as **cetrimide agar** for isolation from samples with mixed flora.

Pigment production *P. aeruginosa* produces a number of pigments.

- **Pyocyanin** is a bluish-green phenazine pigment soluble in water and chloroform [pyocyanin is produced only by *P. aeruginosa* (Fig. 32.1)]. This is enhanced by King A medium.

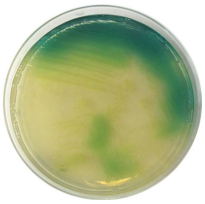


Fig. 32.1 *P. aeruginosa* on nutrient agar showing green pigment production (pyocyanin)

- **Fluorescein** (pyoverdin) is a greenish-yellow pigment soluble in water but not in chloroform. King B medium enhances fluorescein production. This pigment may be produced by many other species.
- **Pyorubin** (red) and **pyomelanin** (brown) in various combinations are produced by some species and are sometimes seen in clinical isolates.

Some strains may be non-pigmented. It is not known whether the pigments have any role in pathogenesis. Some of the pigments, particularly pyocyanin, inhibit the growth of many other bacteria and may, therefore, contribute to *P. aeruginosa* emerging as the dominant bacterium in mixed infections.

2. **Biochemical reactions** *P. aeruginosa* gives a prompt oxidase reaction and is actively motile. Glucose is utilised oxidatively, forming only acid. Hugh and Leifson's **(oxidative fermentative OF**) medium with glucose is used to test the oxidative breakdown of the carbohydrate. Citrate is utilised and nitrate is reduced to nitrite and further, to gaseous nitrogen.

3. **Typing** For epidemiological purposes and to trace the source of infection, *Pseudomonas* isolates can be typed by several methods.

- **Pyocine typing** Depends on the pigment pyocine (this method is obsolete now).
- **Phage typing** This is dependent on specific bacteriophages which lyse only certain pseudomonas. This method was used to clone hospital isolates. Some laboratories still use it.
- **Molecular typing methods** These are widely used now due to their discriminatory power and ease. Two methods commonly used are:
    - Multilocus sequence typing (MLST)
    - Pulsed-field gel electrophoresis (PFGE)

Control

Prevention of *P. aeruginosa* cross-infection in hospitals requires constant vigilance and strict attention to asepsis.

Treatment

Specific antibacterial therapy constitutes only one aspect of the management of serious pseudomonas infections.

Resistance to antimicrobials Antibiotic treatment options are limited as the strains in the hospital are **multidrug-resistant** and now even **pan-drug-resistant.** They may be susceptible to colistin (or amphotericin), which is

tested by E-test (Fig. 32.2a and b).

Occasional opportunistic infections may be caused by a few other species, such as *P. fluorescens, P. putida* and *P. stutzeri.*

Some of the other emerging non-fermenters of clinical importance are increasingly being identified by automated ID systems (e.g., VITEK system and MALDI-ToF for identifying bacteria).These systems are replacing conventional biochemical tests which are time-consuming and require strict quality control of the tests.

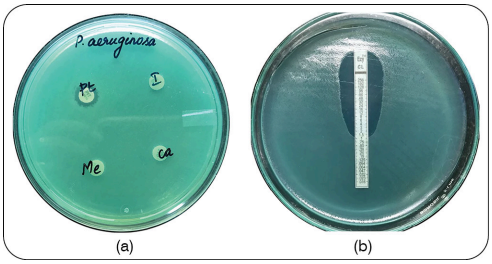


Fig. 32.2 *P. aeruginosa:* (a) multidrug-resistant and (b)susceptible only to colistin (MIC of colistin by E-test) (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

STENOTROPHOMONAS MALTOPHILA

This is a saprophyte and opportunistic pathogen that causes wound infection, urinary tract infection and septicemia in health care settings. It is usually oxidase-negative and acidifies maltose in addition to glucose, lactose and sucrose. The organism is sensitive to cotrimoxazole and resistant to carbapenems.

ELIZABETHKINGIA MENINGOSEPTICUM

Earlier known as *Flavobacterium meningosepticum*, this organism is one of the non-fermenters found in hospital water sinks, moist environments and in infusion salines and humidifiers in neonatal care units. *E. meningosepticum* has been associated with meningitis and sepsis in the newborn. In adults, it causes dialysis-associated peritonitis, septic arthritis, infections in extensive burn wounds, and community-acquired necrotising fasciitis. It grows on enriched media like blood and chocolate agar, and is resistant to several antibiotics.

ALCALIGENES AND ACHROMOBACTER

These non-fermenters are opportunistic pathogens that infect immunocompromised individuals and those on chemotherapy for malignancy. They can cause pneumonia, septicemia, peritonitis and urinary tract infections in these groups of patients. Laboratory diagnosis depends on isolating the organisms which grow on blood agar and are inert biochemically.

BURKHOLDERIA

Burkholderia cepacia

B. cepacia is increasingly being recognised as an opportunist pathogen, particularly for those with cystic fibrosis or chronic granulomatous disease, in whom it causes fatal necrotising pneumonia. It is nutritionally very versatile. It can grow in many common disinfectants and can even use penicillin G as its sole source of carbon. It is oxidase-positive and oxidatively breaks down mannitol, sorbitol and sucrose. It can cause urinary, respiratory and wound infections, peritonitis, endocarditis and septicemia. **It is inherently resistant to most antibiotics.**

Burkholderia pseudomallei

This is the causative agent of **melioidosis**, a glanders-like disease, epizootic in rodents in Southeast Asia, India and North Australia. The disease came into prominence after the Vietnam war and hence is called **Vietnam time-bomb disease**. In India, cases are reported from the western and southern belts. The infections are seen to spike following the rainy season.

The name melioidosis is derived from *melis*, a disease of asses (glanders; see next section), and *eidos,* meaning resemblance. It resembles *B. mallei* but differs in its biochemical characters and motility.

Two thermolabile exotoxins, one **lethal** and the other **necrotising**, have been identified in culture filtrates.

Infection is usually acquired by contamination of wounds and abrasions with soil and water containing the organism. Underlying disease (like diabetes mellitus and chronic liver disease) may increase the risk. It may take many years for the infection to manifest. The human disease may take

two forms:

i) **Acute** It may be a generalised infection presenting as acute septicemia, a subacute typhoid-like disease or pneumonia with hemoptysis resembling tuberculosis. Acute melioidosis has a high case fatality rate.

ii) **Chronic** In the chronic form, there may be multiple caseous or suppurative foci, with abscess formation in the skin and subcutaneous tissues, bones and internal organs.

Long latency and reactivation may occur as the bacillus can survive intracellularly in the reticuloendothelial system. The bacillus has been isolated from water and soil in endemic areas. It is a soil saprophyte that causes infection in rodents and humans accidentally. Human infection occurs commonly through skin abrasions or by inhalation.

Laboratory Diagnosis

Diagnosis is made when there is a high index of clinical suspicion with a history of contact with soil (as is the case with farmers and agricultural workers) followed by an ultrasound examination which may reveal multiple abscesses in the internal organs.

1. **Microscopy** Presumptive identification is made by the demonstration of the bacillus in (Fig. 32.3) exudates by microscopy (small, irregularly staining, gram-negative bacilli, showing typical bipolar '**safety-pin appearance**' with methylene blue stain).

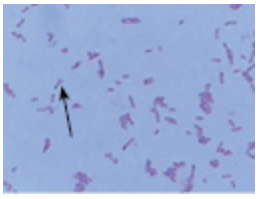


Fig. 32.3 *B. pseudomallei* with bipolar 'safety-pin appearance' on staining with methylene blue stain

2. **Culture** Isolation by culture from sputum, pus, blood or urine shows dry, wrinkled colonies on blood and MacConkey agars. A selective medium used to isolate

B. pseudomallei is Ashdown's selective agar (ASA) medium. It contains gentamicin and crystal violet to suppress other bacteria (useful in sputum

culture).

3. **Biochemical tests** *B. pseudomallei* is commonly identified by its non-fermentative character, oxidase positivity, esculin hydrolysis and reduction of nitrate. One of the tests to screen the organism is its intrinsic resistance to polymixin B which is used as a laboratory diagnostic marker.

PCR test or an automated VITEK system can help identify the organism.

4. **Serology** This does not have much role in diagnosis.

Treatment

Carbapenems, imipenem, meropenem and ceftazidime are the drugs of choice in the acute phase of the disease. For maintenance therapy, cotrimoxazole, tetracycline or amoxicillin-clavulanate are required for many months to prevent recurrence.

Burkholderia mallei

It is the causative agent of **glanders** (*malleus*, in Latin), a disease primarily of equine animals—horses, mules and asses—but capable of being transmitted to other animals and to human beings.

Burkholderia mallei is a slender, non-motile, gram-negative bacillus, 2–5 × 0.5 µm in size, staining irregularly and often having a beaded appearance. It is an aerobe and facultative anaerobe, growing on ordinary media under a wide temperature range. Colonies which are small and translucent initially, become yellowish and opaque on ageing. This organism is quite inactive biochemically, attacking only glucose.

- Human infection is usually **occupational**, found among ostlers, grooms and veterinarians.
- It may be acute or chronic and is protean in character, with localisation in the respiratory tract, skin or subcutaneous tissues.
- In acute glanders, there is fever, mucopurulent nasal discharge and severe prostration. The fatality rate is high.
- While human infection is acquired only rarely from infected animals, laboratory cultures are highly infectious and *B. mallei* is one of the most dangerous bacteria to work with.

ACINETOBACTER

The genus *Acinetobacter* contains strictly aerobic, gram-negative, **coccobacillary** organisms (Fig. 32.4) that are non-motile, oxidase-negative, nitrate-negative and do not ferment sugars. They are 1−1.5 × 1.5−2.5 ∝m in size, often appearing in pairs, mimicking neisseriae in appearance. They grow on routine laboratory culture media.

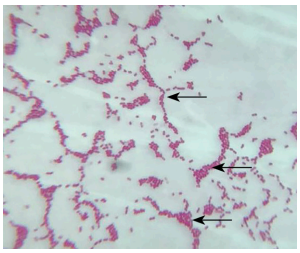


Fig. 32.4 *A. baumannii:* gram-negative coccobacillary forms (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Clinical Case 2

A 30-year-old male was admitted to the emergency department following a road traffic accident with polytrauma and head injury and a Glasgow Coma Scale (GCS) of 5/15. He was sedated, intubated, mechanically ventilated and hemodynamically stabilised. Baseline laboratory parameters were normal. On the fourth day of admission, he developed high-grade fever, increased tracheal secretions with leucocytosis, and procalcitonin level of 4 ng/mL. In addition, his chest X-ray was suggestive of bronchopneumonia. He was diagnosed with ventilator-associated pneumonia (VAP). A broncho-alveolar lavage culture grew gram-negative coccobacilli which was oxidase-negative and did not ferment sugars; this was identified as Acinetobacter baumannii*. It was resistant to all antibiotics except carbapenems and colistin.*

Classification

The classification of *Acinetobacter* has undergone many changes. Currently, using DNA hybridisation studies, they have been assigned to a different DNA homology group. Within the genus *Acinetobacter*, strains commonly isolated in clinical laboratories are called the *Acinetobacter calcoaceticus-baumannii* complex, which is subdivided as follows:

- ***A. baumannii*** as clinical strains, glucose metabolised oxidatively, and citrate-positive

- ***A. lwoffii*** as glucose-negative, citrate-negative, non-hemolytic strain
- ***A. hemolyticus*** as the hemolytic strain

Laboratory Diagnosis

Acinetobacter baumannii forms pinkish colonies (not clearly lactose-fermenting, often referred to as query LF colonies) on MacConkey medium (Fig. 32.5). It can survive for long periods in the hospital environment and colonise almost all patients on prolonged hospitalisation. *Acinetobacter baumannii* produces biofilms in devices and hence is associated with device-related infections. It is listed as the most important pathogen in hospitals. It is an opportunistic pathogen responsible for a wide spectrum of infections. The hospital strains are also **multidrug-resistant** (Fig. 32.6), thus pose a great therapeutic challenge (*Clinical case* 2). Often, colistin is the only antibiotic to which the organism is susceptible. Unlike the standard procedure for testing, **colistin** has to be tested by the **microbroth dilution test** to detect the exact cutoff for susceptibility.

Fig. 32.5 *Acinetobacter baumannii:* query lactose-forming colonies on MacConkey agar plate (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Fig. 32.6 Multidrug-resistant *A. baumannii*; antibiotic susceptibility plate (*Source:*

Health care-associated infections caused by *A. baumannii* are:

- Ventilator-associated pneumonia **(VAP)**
- Meningitis (in patients with shunt)
- Bacteremia (catheter-related bloodstream infection—**CRBSI**)

Treatment

These organisms have acquired resistance to cephalosporins, quinolones, aminoglycosides and in some cases, carbapenems. *A. baumannii* has become problematic in intensive care units (ICUs) because it has developed resistance to broad-spectrum antibiotics, and the incidence of **multidrug-resistant**
A. baumannii **(MDRAB)** infections have continued to increase worldwide. Prevention of health care-associated infections by following standard precautions is the best preventive strategy to control these infections. Doing so will indirectly reduce colonisation and antibiotic use.

Key points

- Members of the genus *Pseudomonas* are rod-shaped bacteria which are motile, catalase and oxidase-positive, gram-negative and obligate aerobes.
- Some species produce pigments—pyocyanine, pyorubrin and pyoverdin—some of which are fluorescent.
- The most important pathogenic species is *Pseudomonas aeruginosa*.
- *Burkholderia cepacia* (the cause of cepacia disease), *Burkholderia mallei* (the cause of glanders) and *Burkholderia pseudomallei* (the cause of melioidosis) are also pathogens.
- *Pseudomonas aeruginosa* causes opportunistic infections of the lung (in cystic fibrosis) and nosocomial infections.
- Factors promoting infection by *Pseudomonas aeruginosa* are bacterial pili, exotoxin A, lipopolysaccharide (cell wall) and the alginate glycocalyx. The ability to form biofilms is also a virulence factor.
- For diagnosis, culture of colonies shows the presence of blue pyocyanin and/or yellow pyoverdin. The bacteria are capable of growing at 42°C.
- *Burkholderia cepacia* can cause severe infections in patients of cystic fibrosis.
- Melioidosis is a pyogenic or granulomatous infection caused by *Burkholderia pseudomallei* which has a safety-pin appearance. It is endemic to some parts of India.

- Glanders is a disease of animals that can be transmitted to humans; it is caused by *Burkholderia mallei*.
- *Acinetobacter baumannii* is a gram-negative coccobacillary form which is associated with multidrug resistance involved in health care- associated infections: VAP, CAUTI and CRBSI.

Questions

Short Answers

1. Antibiotic resistance in *Pseudomonas aeruginosa*
2. Virulence factors of *Pseudomonas aeruginosa*
3. Melioidosis
4. Role of *Pseudomonas aeruginosa* in hospital infections
5. *Burkholderia* infections
6. Health care-associated infections due to multidrug-resistant *Acinetobacter baumannii*

Part III: Bacteriology

Chapter 33

Haemophilus

Chapter navigation

- *Haemophilus influenzae*
- Classification
- Pathogenesis
- Epidemiology
- Vaccines

The genus *Haemophilus* includes a number of species of organisms that are small, pleomorphic, oxidase-positive, fastidious, non-motile and non-sporing. They are gram-negative coccobacilli that can cause infection in humans and animals. They require two accessory growth factors (X or hemin and V or NAD) that are present in blood.

H. influenzae is the first free-living organism whose complete genome has been sequenced. The organism was described by Pfeiffer from sputum of patients with influenza (hence named as **influenza bacillus** or **Pfeiffer's bacillus**).

HAEMOPHILUS INFLUENZAE

Clinical Case

A six-month-old child was brought to the pediatrics outpatient department with the chief complaint of fever, inability to feed and seizures since the morning. On examination, the child was found to have altered sensorium and neck rigidity. At the hospital, cerebrospinal fluid (CSF) obtained through lumbar puncture had a cloudy appearance with a white blood cell (WBC) count of 2,942/mm^3, 88% polymorphonuclear cells, glucose 5 mg/dL, and protein 196 mg/dL suggestive of pyogenic meningitis. Gram-stain of CSF showed gram-negative filamentous and thin bacilli. Antigen detection by latex agglutination was positive for Haemophilus influenzae *type b and the culture on the next day showed a growth on chocolate agar identified as* H. influenzae*. The child responded to treatment with ceftriaxone.*

Morphology

H. influenzae is a small (1.0 × 0.3 μm), gram-negative bacillus, exhibiting considerable pleomorphism. In sputum, it usually occurs as clusters of coccobacillary forms, while in CSF from meningitis cases, long, bacillary and filamentous forms predominate. Cells from young cultures (18–24 hours) are usually coccobacillary, while older cultures are distinctly pleomorphic. **Strains isolated from acute infections are often capsulated.**

Antigenic Properties

The major antigenic determinant of capsulated strains is the **capsular polysaccharide**. *H. influenzae* strains have been classified by Pittman into six capsular types—**a to f.** Typing is done by the agglutination of type-specific antisera. *H. influenzae* type b (Hib) is the most common type associated with infection. **Capsular typing is of medical importance because about 95 per cent of *H. influenzae* isolates from acute invasive infections such as meningitis belong to type b.**

The type b capsular polysaccharide has a unique chemical structure, containing the pentose sugars ribose and ribitol instead of hexoses and hexosamines as in the other five serotypes.

- **Polyribosyl ribitol phosphate (PRP) antigen of Hib capsule** induces IgG, IgM and IgA antibodies, which are bactericidal, opsonic and protective. **Hib PRP is used for immunisation**.
- *H. influenzae* strains lacking a capsule cannot be typed and are called **non-typeable strains**. After Hib, the non-typeable strains are the most relevant in clinical infections.
- The **outer membrane protein antigens (OMP)** show considerable variation. OMP antigens of Hib have been classified into at least 13 subtypes.
- *H. influenzae* **lipooligosaccharides (LOS)** are antigenically complex. OMP and LOS subtyping may be of epidemiological value.

Epidemiology

There is considerable similarity between the epidemiology of *H. influenzae*

and *S. pneumoniae*. Both are indigenous to human beings, primarily colonising the upper respiratory tract. Infection is transmitted by the respiratory route. Carriage in the upper respiratory tract is common, particularly in young children, but such strains are usually non-capsulated and not responsible for acute invasive infection. Maternal antibodies are protective and can be transmitted from mother to child. Non-immunised children between six months and two years of age become susceptible when maternal antibody wanes. Immunity is type-specific. With the increased use of the Hib vaccine, infections due to *H. influenzae* type b are decreasing.

Pathogenicity

H. influenzae is an exclusively human pathogen. Diseases caused by *H. influenzae* may be categorised into two groups:

i) ***Invasive*** *H. influenzae* is the primary pathogen which causes acute invasive infections. It spreads through blood, being protected from phagocytes by its capsule. **Haemophilus meningitis** is the most important infection in this group, others being bacteremia, pneumonia, laryngoepiglottitis, conjunctivitis, arthritis, endocarditis and pericarditis. These infections are usually seen in children and are caused by the capsulated strains, type B accounting for most cases. *H. influenza*e along with pneumococcus is the primary cause of childhood pneumonia and meningitis in the developing world.

ii) ***Non-invasive*** The organism spreads locally from the throat (where it is a commensal) along mucosal surfaces and causes secondary infections of the respiratory tract including otitis media, sinusitis and exacerbations of chronic bronchitis and bronchiectasis. These are usually seen in adults and are often caused by the non-capsulated strains.

Clinical Presentation

- ***Meningitis*** This is the most serious disease produced by *H. influenzae*, with case fatality rates up to 90 per cent if untreated. The bacilli reach the meninges from the nasopharynx and through the bloodstream. This disease is more **common in children between two months and three years of age** (*Clinical*

case). This age incidence has been correlated with the absence of bactericidal anti-PRP antibodies. Older children develop immunity as a result of subclinical infection. It has been reported that in the tropics, non-type B strains may be responsible for meningitis more often than in the temperate zones.

- ***Laryngoepiglottitis (croup)*** This is an acute inflammation of the epiglottis with obstructive laryngitis seen in children above two years. Untreated cases may be fatal within hours.
- ***Pneumonia*** *Haemophilus pneumonia* occurs in infants and is accompanied by empyema and sometimes meningitis as well. In older children and adults, lobar pneumonia is produced. While these are primary infections caused by capsulated strains**, bronchopneumonia may occur as a secondary infection with the non-capsulated strains**.
- ***Bronchitis*** *H. influenzae* is an important pathogen associated with pneumococci in acute exacerbations of chronic bronchitis and bronchiectasis.
- ***Suppurative lesions*** Arthritis, endocarditis and pericarditis may result from hematogenous dissemination. Otitis media occurs by direct spread from the nasopharynx. Cellulitis, particularly in the buccal and periorbital areas, is seen in young children.

Laboratory Diagnosis

1. **Specimen** **CSF, blood** or **sputum** is collected depending on the site of infection. As the bacillus is very sensitive to low temperatures, specimens should never be refrigerated before inoculation. Blood cultures are often positive in cases of laryngoepiglottitis and pneumonia. Sputum is not an ideal specimen to isolate the organism in cases of pneumonia.

2. **Microscopy** In meningitis, the presence in CSF of pleomorphic, gram-negative bacilli should arouse the suspicion of *H. influenzae* infection.

3. **Direct antigen detection** The capsular polysaccharide antigen may be present in CSF in meningitis and in urine in systemic infection. Latex particle agglutination for the detection of type b antigen is useful in rapid diagnosis.

4. **Culture Early culture on one of the following culture media is vital for isolation as the fastidious organism dies at room temperature:**

- **Chocolate agar**—when blood agar is heated to 80–90°C for 15–20 minutes, the V factor is released from within the erythrocytes
- **Blood agar** with *Staphylococcus aureus* streaked across the plate, as seen in Fig. 33.1a

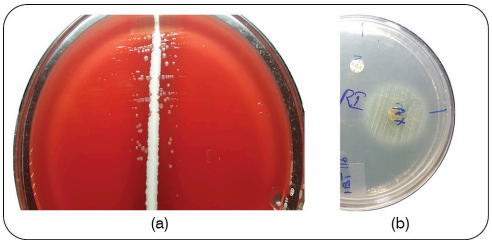


Fig. 33.1 *H. influenzae:* (a) on blood agar showing satellitism around *S. aureus* streaks and (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- **Nutrient agar** with discs of X and V factors
- **Levinthal's medium** is a mixture of blood and nutrient broth on which iridescence may be demonstrated on Levinthal's medium
- **Filde's agar** is best for primary isolation of *H. influenzae* and gives copious growth due to peptic digest and blood

The media should be incubated in an environment of 5–10% CO_2 and high humidity. After overnight incubation at 37°C, small opaque colonies of gram-negative, short, oxidase-positive cocci appear. Typing may be done by slide agglutination using specific antisera.

5. **Cultural characteristics** The organisms require accessory growth factors present in blood which are essential for growth (Table 33.1).

Table 33.1 Growth characteristics of the *Haemophilus* species

Species	Growth requirements			Hemolysis on horse blood agar
	X	V	CO_2	

H. influenzae	+	+	–	–
H. aegyptius	+	+	–	–
H. ducreyi	+	–	Variable	Variable
H. parainfluenzae	–	+	–	–
H. haemolyticus	+	+	–	+
H. parahaemolyticus	–	+	–	+
H. aphrophilus	+	–	+	–
H. paraphrophilus	–	+	+	–

i) **X factor** is hemin or other porphyrins required for the synthesis of cytochrome and other heme enzymes such as catalase and peroxidase involved in aerobic respiration. It is heat-stable.

ii) **V factor** is a co-enzyme, nicotinamide adenine dinucleotide (NAD) or NAD phosphate (NADP), which acts as a hydrogen acceptor in the metabolism of the cell. It is heat-labile and gets destroyed at 120°C in a few minutes. It is present in red blood cells and in many other animal and plant cells. It is synthesised by some fungi and bacteria (e.g., *S. aureus*) and released into the surrounding medium. *H. influenzae* grows well on blood agar in which a source of the V factor is also provided.

Satellitism
Colonies show satellitism when grown near *S. aureus*. This demonstrates the dependence of *H. influenzae* on the V factor, which is available in high concentrations near staphylococcal growth. Another differentiating test is growth around discs incorporated with X, V and XV factor. This helps in differentiating the species of *Haemophilus* (Fig. 33.1b).

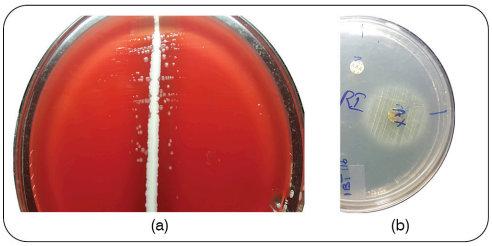


Fig. 33.1 *H. influenzae:* (b) requirement of XV factor (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

6. **Biochemical tests** These tests are not routinely done for identification

due to their exacting nature. *H. influenzae* ferment glucose and xylose without production of gas.

7. **Biotyping** This has been done on the basis of indole production, and urease and ornithine decarboxylase activity. **Eight biotypes** have been identified, of which biotype I is most frequently responsible for meningitis.

Treatment

Cefotaxime or ceftriaxone are the drugs of choice for the treatment of haemophilus meningitis. Ampicillin and cotrimoxazole were popular for respiratory infections, but as plasmid-borne resistance to these drugs is now common, amoxicillin–clavulanate or clarithromycin is now considered more effective.

Prevention

Active immunisation with the **Hib PRP** vaccine is indicated as serious infections are caused by type b strains. Purified PRP is immunogenic in older children and adults. However, as with other polysaccharide antigens, PRP is poorly immunogenic in children below the age of two years. **Its immunogenicity has been improved by coupling it with protein carriers like diphtheria and tetanus toxoids or meningococcus outer membrane protein.** These are called **conjugate vaccines**. Such conjugate Hib PRP vaccines are available for use in young children and can be given along with DPT immunisation.

HAEMOPHILUS AEGYPTIUS

Recent DNA studies have shown that the bacillus is identical to non-capsulated *H. influenzae.* Therefore, it is now named *H. influenzae* biotype *aegyptius.* It belongs to *H. influenzae* biotype III. It is worldwide in distribution and causes a highly contagious form of conjunctivitis (‘**pink eye**’). It is especially common in the tropics and subtropics and may occur in epidemic form. It responds to local sulphonamides or gentamicin.

It has also been identified as the causative agent of **Brazilian purpuric fever (BPF)**, endemic in South America in which conjunctivitis proceeds to fulminant septicemia in infants and children with high fatality.

HAEMOPHILUS DUCREYI

H. ducreyi is a short, ovoid gram-negative bacillus (1–1.5 μm × 0.6 μm) with a tendency to occur in end-to-end pairs or short chains. It appears as groups or whorls or in parallel chains, giving a '**school of fish**' or '**rail road track**' appearance. It can be grown on fresh clotted rabbit blood. It may also be grown on the chorioallantoic membrane of the chick embryo and on chocolate agar (enriched with IsoVitaleX, which is a nutritional factor, fetal calf serum and vancomycin). *H. ducreyi* forms small, grey, translucent colonies after incubation at 35°C under 10% CO_2 and high humidity in 2–8 days. Smears made after 24–48 hours' incubation show tangled chains of bacilli.

Infection

H. ducreyi causes **chancroid** or **soft sore** which is a sexually transmitted infection. It is characterised by tender, non-indurated irregular ulcers on the genitalia. This infection remains localised, spreading only to the regional lymph nodes which are enlarged and painful. There is no immunity following infection but hypersensitivity results, resulting in a positive intradermal test with killed bacilli.

Treatment

H. ducreyi may be treated with erythromycin, cotrimoxazole, ciprofloxacin or ceftriaxone.

HAEMOPHILUS PARAINFLUENZAE

It is a commensal in the upper respiratory tract and has been reported to cause subacute bacterial endocarditis, urethritis and acute pharyngitis. It differs from
H. influenzae in requiring only the V factor and not the X factor for growth.

HACEK GROUP OF ORGANISMS

They are a small group of heterogeneous bacteria which are fastidious, gram-negative organisms, located in the upper respiratory tract (mouth, throat and lungs), gastrointestinal tract and urinary tract. Although HACEK

bacteria are usually not associated with any problem, they have been known to cause infection in some cases, especially prosthetic heart valve-associated infective endocarditis.

The **HACEK** group of bacteria include:

- *Haemophilus* species
- *Aggregatibacter* species
- *Cardiobacterium hominis*
- *Eikenella corrodens*
- *Kingella* species

Infections

The infections caused by this group of organisms include:

- Endocarditis (infection of the inner lining and valves of the heart)
- Lung and respiratory infections
- Gum infections
- Bone infections
- Urinary tract infections
- Abscesses

As these are fastidious organisms, their isolation is often difficult. They are slow-growing and demand nutritional supplements and a carbon dioxide-enriched environment. Blood cultures take about 7–30 days to get a positive yield.

Treatment

Native valve endocarditis is often treated with penicillin and gentamicin for synergistic action. The duration of treatment is four weeks and is usually successful. Cefotaxime or ceftriaxone with or without an aminoglycoside or rifampin may be optimal therapy for endocarditis. Oral ciprofloxacin also has been used successfully. Treatment of prosthetic valve endocarditis is more complicated, requiring surgical intervention and antibiotics for a prolonged period of time.

AGGREGATIBACTER

Aggregatibacter (formerly known as *Actinobacillus*) *actinomycetemcomitans* is a small, fastidious, gram-negative coccobacillus, which causes infective endocarditis and periodontal infection. It is part of the normal flora of the mouth, especially the gingival and supragingival crevices.

CARDIOBACTERIUM

Cardiobacterium hominis is the major species in this group. It is a gram-negative, pleomorphic bacillus which occurs commonly as a commensal in the human nose and throat, and may cause endocarditis, particularly in those with pre-existing cardiovascular disease. It grows on blood agar under 3–5% CO_2 and high humidity. It ferments a wide range of sugars, forms indole and is oxidase-positive, but catalase- and nitrate-negative. It is sensitive to many antibiotics, penicillin and streptomycin being the recommended drugs.

KINGELLA

The genus *Kingella*, comprising some species of oxidase-positive, non-motile, gram-negative rods, with a tendency to occur as coccobacillary and diplococcal forms, was formerly grouped under the genus *Moraxella*. Organisms belonging to this genus are part of the normal oral flora. *K. kingae* has been associated with endocarditis and infections of the bones, joints and tendons.

EIKENELLA CORRODENS

This is an oxidase-positive, facultatively anaerobic, capnophilic, gram-negative bacillus. The name ‘corrodens’ refers to the characteristic pitting or corroding of blood agar by colonies of the bacterium. It is present in the mouth, upper respiratory tract and gastrointestinal tract of human beings. Infection follows salivary or a fecal contamination and usually involves the skin and subcutaneous tissues, though rarely, osteomyelitis, pneumonia, endocarditis and meningitis may occur. It is sensitive to penicillin and tetracycline.

Key points

- Members of the genus *Haemophilus* are gram-negative coccobacilli that are non-motile, facultative anaerobes, positive by the catalase and oxidase tests. They require either hemin (X factor) or co-enzyme 1 (V factor) or both for growth. This can be demonstrated by satellitism.
- *Haemophilus influenzae* is the most important species; *Haemophilus ducreyi* and other species may also cause disease in humans.
- The organism can be typed serologically according to capsular antigens, and type b (Hib) is often involved in acute infections. Encapsulated type B strains cause meningitis, epiglottitis in children below the age of two years, conjunctivitis and cellulitis.
- Non-encapsulated strains of *H. influenzae* are often found in the healthy respiratory tract but can cause middle ear infections in children and pneumonia in compromised adults.
- For diagnosis, samples of blood or CSF are inoculated onto chocolate agar or other media containing X and V factors. *H. influenzae* colonies exhibit satellitism on blood agar plates when grown in the vicinity of *Staphylococcus aureus* colonies.
- Detection of Hib capsular polysaccharide in CSF by latex agglutination is a means of establishing rapid diagnosis of *H. influenzae* meningitis.
- Routine childhood vaccination with Hib conjugate vaccine has almost eliminated invasive Hib disease in developed nations.
- *Haemophilus influenzae* biogroup *aegyptius* causes a purulent conjunctivitis.
- *Haemophilus ducreyi* causes soft chancre (chancroid). This disease is sexually transmitted and manifests as a painful, ulcerative lesion on the genitalia. Such ulcers may predispose to the acquisition of infection with HIV.
- HACEK is a group of fastidious, slow-growing bacteria that can cause severe infections, particularly endocarditis. Blood cultures from infected patients take 7–30 days to become positive.

Questions

Essay

1. Describe the pathogenesis and laboratory diagnosis of *H. influenzae* meningitis.

Short Answers

1. Hib vaccine
2. Satellitism in *H. influenzae*
3. Methods of culture and identification of *H. influenzae*
4. Pathogenesis and identification of *H. ducreyi*

Part III: Bacteriology

Chapter 34

Brucella and Bordetella

Chapter navigation

- *Brucella*
  - Classification
  - Pathogenesis
- *Bordetella*

BRUCELLA

The genus *Brucella* consists of very small, non-motile, aerobic, gram-negative coccobacilli that grow poorly on ordinary media and have little or no fermentative powers. Brucella species cause a zoonotic infection in humans called **brucellosis**. The organism primarily affects goats, sheep, cattle, buffaloes, pigs and other animals and is transmitted to humans by contact with infected animals or through their products. The human disease was recognised along the Mediterranean and is known by various names such as **Mediterranean fever**, **Malta fever** and **undulant fever**. The bacteria was named after a British army doctor, David Bruce who discovered *Brucella melitensis* (*Brucella* after Bruce*; melitensis* is the Roman name for Malta). Subsequently, *B. abortus* and
B. suis were isolated by Bang and Traum from cows and pigs respectively. These three species cause human brucellosis.

Other species causing animal infections include *B. canis,* isolated from cases of canine abortion, *B. ovis* from abortion in sheep and
B. neotomae from desert woodrats. *B. canis* may occasionally cause a mild human disease, but the other two are not pathogenic for humans.

Clinical Case 1

A 50-year-old farmer presented with a history of intermittent fever for the past three weeks with loss of

appetite and nausea. The farmer sold milk from his cattle at the market nearby. On examination, no specific signs were found. Detailed laboratory investigation revealed only anemia. Given his occupational history, blood culture and standard agglutination test (SAT) for antibodies were carried out. The culture at this time was negative but SAT titres were >1:1,280, suggestive of brucellosis. The patient responded to a course of doxycycline and streptomycin.

Note: *Please note the clinical presentation with non-specific signs and symptoms. Diagnosis depends on a high degree of suspicion in a patient of pyrexia of unknown origin with a suggestive occupational history and a positive specific tests for brucellosis.*

Morphology

Brucellae are gram-negative, coccobacilli or short rods, 0.5–0.7 × 0.6–1.5 μm in size. (Fig. 34.1). They may be mistaken for cocci (as was done by Bruce, who called them *Micrococcus melitensis*). In older cultures, they may show irregular forms. They are non-motile, non-capsulated and non-sporing.

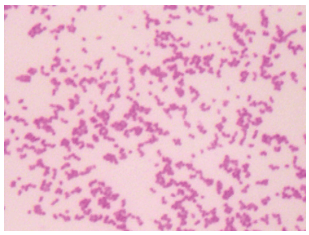


Fig. 34.1 *Brucella*: gram-negative coccobacilli (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Classification

Based on nucleic acid homology and gene sequencing, the genus *Brucella* is considered monospecific. However, due to their differences in pathogenicity and natural hosts, the traditional classification has been largely retained. There are ten species that comprise the genus. The organisms most commonly causing human infection and their natural hosts are: *B. melitensis* (goats and sheep),
B. suis (swine), *B. abortus* (cattle) and *B. canis* (dogs).

A second classification of brucellae into two main species—
B. melitensis and *B. abortus*—is based on their CO_2 requirements, H_2S production, sensitivity to dyes (basic fuchsin and thionin), agglutination by monospecific sera, phage lysis and oxidative metabolic tests with amino acids and carbohydrates. Many biotypes have been recognised in these species. *B. suis* strains that produce H_2S are known as **'American' strains** and those that do not as **'Danish' strains.**

Antigenic Structure

The somatic antigens of brucellae contain two main antigenic determinants, **A and M,** which are present in different amounts in the three major species. *B. abortus* contains predominantly A, while *B. melitensis* contains M. *B. suis* has an intermediate antigenic pattern. Major antibody component, absorbed A and M monospecific sera are useful for species identification by the agglutination test.

Antigenic cross-reactions exist between brucellae and *Vibrio cholerae* and persons receiving the cholera vaccine may develop *Brucella* agglutinins lasting for about three years. Antigenic cross-reactions also exist with *Escherichia coli* O:116; O:157, Salmonella serotypes group N (O:30 antigen Kauffman and White), *Pseudomonas maltophilia, Yersinia enterocolitica* and *Francisella tularensis.* A superficial L antigen resembling the *Salmonella* Vi antigen has been described.

Virulence Factors

The virulence factors of brucellae are the following:

- Lipopolysaccharides on its cell wall, especially the O-polysaccharides (O-PS) of the LPS play a major role in virulence.
- The binding and penetration of the organism into the host cells is regulated by the genes *BvrR/BvrS*.
- The ability to inhibit fusion of phagosomes and lysosomes and replication in the phagosomes is a second virulence mechanism.
- Other regulatory genes for virulence are *VirB1–VirB12,* which facilitate the bacterial intracellular growth in phagocytic and non-phagocytic cells.

***Brucella* bacteriophage** Several bacteriophages that lyse the *Brucella* strains have been isolated. These phages are serologically similar. The **Tbilisi (Tb) phage has been designated as the reference phage,** and at routine test dilution (RTD), lyses only *B. abortus. B. suis* is lysed at 10,000 RTD, while *B. melitensis* is not lysed at all.

Resistance

Brucellae are destroyed by heat at 60°C in 10 minutes and by 1% phenol in 15 minutes. They are killed by pasteurisation. They remain viable for 10

days in refrigerated milk, one month in ice cream, four months in butter and for varying periods in cheese depending on its pH. They may also survive for many weeks in meat. They tend to die in buttermilk. *B. melitensis* may stay alive for six days in urine, six weeks in dust and ten weeks in water.

> The species identification of *Brucella* strains is not straightforward. Strains that behave biochemically as *abortus* and serologically as *melitensis* and vice versa are often seen. Species and biotype identification depends on a variety of other factors besides antigenic structure.

Pathogenicity

All three major species of brucellae are pathogenic to humans, *B. melitensis* being most pathogenic while *B. abortus* and *B. suis* are intermediately pathogenic.

Brucella is primarily an **intracellular pathogen affecting the reticuloendothelial system** with a predilection for macrophages, dendritic cells (DCs) and trophoblasts. The bacteria can enter, survive and replicate within these cells and cause disease. This accounts for its refractoriness to chemotherapy and the co-existence of viable bacilli with high levels of circulating antibodies. The lipopolysaccharide component of the *Brucella* cell wall is a virulence factor.

Transmission Organisms from the infected animal enter the human body through a wound, the conjunctiva, by inhalation or by ingestion of products from infected animals.

Incubation period This is usually about 10–30 days, but may sometimes be very prolonged.

Spread They spread from the initial site of infection through lymphatic channels to the local lymph glands where they multiply intracellularly. They then spill over into the bloodstream and are disseminated throughout the body. They have a **predilection for the placenta**, probably due to the presence of erythritol in it, which has a stimulating effect on brucellae. Fever, sweats and extreme fatigue occur 2–4 weeks after the initial infection.

Types Human infection may be of three types:

i) Latent infection with only serological but no clinical evidence;
ii) Acute or subacute brucellosis; and
iii) Chronic brucellosis.

Acute brucellosis is generally caused by *B. melitensis.* It is usually known as **undulant fever** but this is misleading as only few cases show the undulant pattern. It is associated with prolonged bacteremia and irregular fever. Symptoms vary with muscular and articular pains, asthmatic attacks, nocturnal drenching sweats, exhaustion, anorexia, constipation, nervous irritability and chills. Common complications are articular, osseous, visceral or neurological. There could be marked thrombocytopenia with low platelet counts, which may result in bleeding or thrombocytopenic purpura.

Chronic brucellosis may be non-bacteremic, with a low-grade infection and periodic exacerbations. The symptoms are generally related to a state of hypersensitivity in the patient. Common clinical manifestations are sweating, lassitude and joint pain with minimal or periodic pyrexia. The illness lasts for years. Due to varied symptoms and prolonged fever, the infection is clinically considered as one of pyrexia of unknown origin (PUO).

Immunity in brucellosis is mainly cell-mediated. The T_h1 type of T helper cell response and cell-mediated immunity are required to eliminate brucellae through activated macrophages; tumour necrosis factors alpha and gamma, and interleukins 1 and 2 are important mediators of the protective response. This is probably the most important mechanism in recovery and immunity in brucellosis.

Tissue reaction to *Brucella* consists of granuloma formation with epithelial cells, giant cells, lymphocytes and plasma cells. Granulomas heal with fibrosis and sometimes become calcified.

Epidemiology

Human brucellosis is acquired from animals, directly or indirectly. Goats, sheep, cattle, buffaloes and swine are the common sources. The modes of infection are ingestion, contact, inhalation or accidental inoculation. Person-to-person spread does not ordinarily occur.

The most important vehicle of infection is raw milk. Milk products, meat from infected animals and raw vegetables or water supplies contaminated by the feces or urine of infected animals may also be responsible. Contact infection is especially important as an occupational hazard in veterinarians, butchers and animal handlers, and is particularly common during the

calving season. I**n various parts of India, most human infections are due to *B. melitensis* acquired from goats and sheep**.

Laboratory Diagnosis

The clinical manifestations of human brucellosis are variable, and only if a high index of suspicion is maintained will the disease be identified. Clinical diagnosis is almost impossible and laboratory confirmation is essential (*Clinical case* 1). Laboratory methods for diagnosis include culture, serology and hypersensitivity tests.

1. **Cultural characteristics** Brucellae are strict aerobes and do not grow anaerobically. *B. abortus* is **capnophilic,** requiring 5–10% CO_2 for growth. The optimum temperature is 37°C (range 20–40°C) and pH is 6.6–7.4.

- Growth is slow and scanty in **ordinary media.** The media currently employed are serum dextrose agar, serum potato infusion agar, trypticase soy agar or tryptose agar. The addition of bacitracin, polymyxin and cycloheximide to the above media makes them selective. Erythritol has an especially stimulating effect on the growth of brucellae.
- Growth on **solid media** shows small, moist, translucent and glistening colonies (Fig. 34.2). Mucoid, smooth and rough types of colonies appear, associated with changes in antigenic structure and virulence.

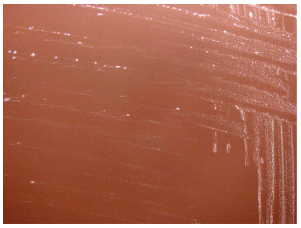


Fig. 34.2 *Brucella suis* colonies on chocolate agar (*Source:* Public Health Image Library, ID 17132 / Todd Parker, Ph.D., Assoc Director for Laboratory Science, Div of Preparedness and Emerging Infections at CDC / CDC)

Blood culture is the most definitive method for the diagnosis of brucellosis. In recent years, the isolation rates of *Brucella* from these specimens has increased due to the automated culture and identification systems.

Bone marrow cultures yield a higher rate of isolation and remain positive long after the blood culture has become negative.

Automated blood culture systems like BacT/ALERT and ID systems like VITEK and MALDI-ToF have greatly reduced the reporting time to within 48 to 72 hours, as compared to two to three weeks required for the conventional *Brucella* broth or Castaneda's methods.

Castaneda's method Blood is inoculated into a bottle of trypticase soy broth or *Brucella* broth in a biphasic blood culture bottle (Fig. 34.3). Cultures are positive only in about 30–50 per cent of cases, even when repeated samples are tested. *B. melitensis* and *B. suis* are isolated more readily than *B. abortus*.

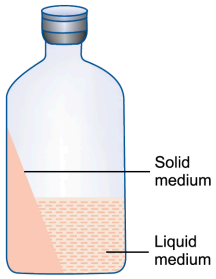


Fig. 34.3 Castaneda's bottle for blood culture

2. **Biochemical reactions** Brucellae are catalase-, oxidase- and urease-positive. No carbohydrates are ordinarily fermented, though they possess oxidative capacity.

3. **Serological methods** These are important in diagnosis. Several serological tests have been developed, including the standard agglutination test (also known as *Brucella* agglutination test) and ELISA.

The **standard agglutination test (SAT)** is commonly done in most laboratories. Equal volumes of serial dilutions of the patient's serum and the standardised antigen (killed suspension of a standard strain of *B. abortus*) are mixed and incubated at 37°C for 24 hours or 50°C for 18 hours.

- **Positive SAT** A titre of 160 or more is considered significant. Most patients with acute brucellosis develop titres of 640 or more in 3–4 weeks of illness. Titres tend to decline after the acute phase of the illness. Both IgM and IgG antibodies appear in 7–10 days after the onset of clinical infection. As the disease progresses, IgM antibodies decline, while IgG antibodies persist

or increase in titre. The IgG can be differentiated from IgM by treating the serum with 2-mercaptoethanol (2ME). IgM is destroyed by this agent. The titre following the treatment with 2ME reflects IgG.

- **False-negative SAT** Sera often contain **'blocking'** or **'non-agglutinating' antibodies**. The blocking effect may sometimes be removed by prior heating of the serum at 55°C for 30 minutes or by using 4% saline as the diluent for the test. The most reliable method for obviating the blocking effect and detecting 'incomplete' antibodies is the antiglobulin (Coombs) test.
- **False-positive SAT**
  - **False positive reaction may occur due to antigenic cross-reaction with several gram-negative bacteria.**
  - People infected by *V. cholerae* and those receiving the cholera vaccine may develop *Brucella* agglutinins lasting for about three years.
  - Infection with any of the following gram-negative organisms can give a false-positive SAT.
    – *Escherichia coli* O:116; O:157
    – *Salmonella* serotypes group N (O:30 antigen Kauffman and White)
    – *Pseudomonas maltophilia*
    – *Yersinia enterocolitica*
    – *Francisella tularensis*
    – *Salmonella* Vi antigen

The **complement fixation test** is more useful in chronic cases as it detects the IgG antibody also. However, this is no longer preferred on account of its cumbersome technique.

ELISA is sensitive and specific and can detect IgM and IgG antibodies separately. It is, therefore, useful for differentiation between acute and chronic infection.

Diagnosis in animals

Rapid methods such as rapid **dipstick test** and **Rose Bengal card test** have been employed for the detection of brucellosis in herds of cattle.

Milk ring test is used to **detect *Brucella* antibodies in milk.** A sample of whole milk is mixed well with a drop of the stained *Brucella* antigen (a concentrated suspension of killed *B. abortus* stained with hematoxylin) and incubated in a water bath at 70°C for 40–50 minutes. If antibodies are present in the milk, the bacilli are agglutinated and rise with the cream to form a blue ring at the top, leaving the milk unstained.

Prophylaxis

As the majority of human infections are acquired by the consumption of contaminated milk, prevention consists of checking dairy animals for brucellosis. No suitable vaccine is available for humans. However, vaccines have been developed for use in animals. *B. abortus* strain 19 vaccine is protective in cattle.

Treatment

Brucellosis in adults is treated with doxycycline for at least 45 days along with streptomycin IM daily for the first two weeks. A complete course is necessary to eradicate the infection. Children are treated with cotrimoxazole with rifampicin or gentamicin.

BORDETELLA

The genus *Bordetella* is named after Jules Bordet. The species responsible for human infection is *Bordetella pertussis* (*pertussis* means intense cough). A related bacillus, *B. parapertussis*, was isolated from mild cases of whooping cough and *B. bronchiseptica* may occasionally infect human beings, producing a condition resembling pertussis.

BORDETELLA PERTUSSIS

Clinical Case 2

A four-month-old child was brought from a peripheral centre in a remote rural area with complaints of severe cough ending with a typical whoop for the past 10–12 days and apnea during the bouts, followed by vomiting. The child was said to be sleeping comfortably between bouts. Blood counts were raised with lymphocytosis. There was no history of immunisation since birth. The laboratory reported growth of B. pertussis *from the nasopharyngeal swab. A diagnosis of whooping cough was made. In the hospital, the child responded well to erythromycin and supportive care.*

Morphology

B. pertussis is a small, ovoid gram-negative coccobacillus of uniform size and shape (Fig. 34.4). It is non-motile and non-sporing. It is capsulated but tends to lose the capsule on repeated cultivation.

Bipolar metachromatic granules may be demonstrated on staining with toluidine blue.

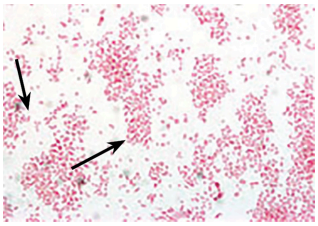


Fig. 34.4 Gram stain of *B. pertussis* showing gram-negative coccobacilli which appear as whorls resembling thumbprints (*Source:* CDC [https://www.cdc.gov/pertussis/clinical/disease-specifics.html])

Virulence Factors

Bordetella pertussis has several virulence factors.

- ***Agglutinogens*** Bordetellae carry surface agglutinogens associated with fimbriae. Strains causing infection belong to types 1, 2 and 3, hence vaccine strains contain these factors. Agglutinogens promote virulence by helping bacteria to attach to respiratory epithelial cells. **They are useful in serotyping strains and in epidemiological studies**.
- ***Filamentous hemagglutinin (FHA)*** This is one of the three hemagglutinins produced by *B. pertussis*, the others being PT and a lipid factor. **It adheres to the cilia of the respiratory epithelium and to erythrocytes. Antibodies to FHA are protective** and **FHA is used in acellular pertussis vaccines** along with PT and pertactin.

  FHA and PT hemagglutinins promote secondary infection by coating other bacteria such as *Haemophilus influenzae* and *S. pneumoniae* and assisting their binding to respiratory epithelium. This phenomenon has been termed **piracy of adhesins.**

- ***Pertactin*** It is an outer membrane protein (OMP) antigen present in all virulent strains of *B. pertussis*. Antibody to pertactin

can be seen in the blood of children after infection or immunisation. **Pertactin is included in acellular pertussis vaccines.**

Toxins

- ***Pertussis toxin (PT)*** This is present only in *B. pertussis*. PT is expressed on the surface of the bacillus and secreted into the surrounding medium. The toxin exhibits diverse biological **lymphocytosis producing factor (LPF)** and causes profound lymphocytosis in pertussis patients. The **histamine-sensitising factor (HSF)** (responsible for heightened sensitivity to histamine) and **islet activating protein (IAP)** which induce excessive insulin secretion by the pancreatic islet cells are seen only in experimental animals and **not** in patients.
    - **PT is a 117,000**-molecular weight hexamer protein composed of six subunits with an A–B structure (A being the enzymatically active moiety and B the binding component). It can be toxoided**. PT toxoid is the major component of acellular pertussis vaccines**.
    - *Bordetella pertussis* strains carry a **novel allele for the pertussis toxin promoter**, which confers increased pertussis toxin (Ptx) production. Epidemiologic data suggest that these strains are more virulent in humans.
- ***Adenylate cyclase (AC)*** Known as the AC toxin (ACT), it acts by catalysing the production of cAMP by various types of cells.
- ***Heat-labile toxin (HLT)*** It is a cytoplasmic protein present in all bordetellae. It is dermonecrotic and lethal in mice. Its pathogenic role is not known.
- ***Tracheal cytotoxin (TCT)*** It is a low-molecular-weight peptidoglycan which induces ciliary damage in hamster trachea. Its role in disease is not known.
- ***Lipopolysaccharide (LPS)*** LPS or the **heat-stable toxin** is present in all bordetellae and exhibits features of gram-negative

bacterial endotoxins. It is present in the whole-cell pertussis vaccine but is not considered to be a protective antigen.

Resistance

It is a delicate organism, killed readily by heat (55°C for 30 minutes), drying and disinfectants. It survives at 0–4°C. Outside the body, *B. pertussis* survives for five days in dried droplets, three days on cloth and a few hours on paper.

Pathogenesis

B. pertussis is an obligate human parasite and is responsible for whooping cough or pertussis in humans. Infection occurs through aerosols and close contact. In the initial stages, the bacilli are confined to the nasopharynx, trachea and bronchi. Clumps of bacilli may be seen enmeshed in the cilia of the respiratory epithelium. As the disease progresses, inflammation extends into the lungs, producing diffuse bronchopneumonia with desquamation of the alveolar epithelium (*Clinical case 2*). After an **incubation period** of about 1–2 weeks, the disease takes a protracted course, comprising **three stages**, each lasting approximately two weeks.

i) **Catarrhal stage** onset is insidious, with low-grade fever, catarrhal symptoms and a dry, irritating cough. Clinical diagnosis in this stage is difficult. This is also the stage of maximum infectivity. (If suspected, treatment can arrest the infection at this stage.)

ii) **Paroxysmal stage** leads to increase in cough intensity and occurs in distinctive bouts. During the paroxysm, the patient experiences violent spasms of continuous coughing, followed by a long in-rush of air into the almost empty lungs, with a characteristic **whoop** (hence the name whooping cough) (Fig. 34.5).

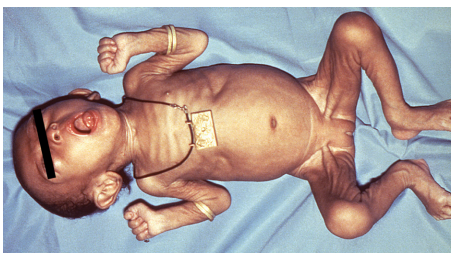


Fig. 34.5 Pertussis in a female infant who presented with paroxysms of cough (notice the

indrawn chest muscles and protruding tongue) (*Source:* Public Health Image Library, ID 6379 / CDC)

iii) **Convalescent stage** follows the paroxysmal stage. Here, the frequency and severity of cough gradually decreases. The disease usually lasts 6–8 weeks, though in some, it may be very protracted.

Complications

The following complications could arise:

- P**ressure effects** (subconjunctival hemorrhage, subcutaneous emphysema)—these occur during the violent bouts of coughing.
- **Respiratory complications** (bronchopneumonia, lung collapse)—these are self-limited, with the atelectasis resolving spontaneously.
- **Neurological complications** (convulsions and coma)—these may result in permanent sequelae such as epilepsy, paralysis, retardation, blindness or deafness.

The infection is limited to the respiratory tract and the bacilli do not invade the bloodstream.

Epidemiology

Whooping cough is predominantly a pediatric disease, the incidence and mortality being highest in the first year of life. **Maternal antibodies do not protect against the disease.** Immunisation should, therefore, be started early. The disease is more common in females than in males at all ages. It is worldwide in distribution. It occurs in epidemic form periodically; the disease is never absent from any community.

Whooping cough is one of the most infectious of bacterial diseases and non-immune contacts seldom escape the disease.

With universal immunisation, childhood pertussis is on the decline but adolescent pertussis is on the rise due to waning of immunity at that age. Adults become prone to infection unless they receive booster immunisation.

Around 1980, pertussis cases started increasing gradually and by 2017, it had risen to 18,000 in the US (CDC). In India, around 25,206 cases of

pertussis were reported in 2015 (EPI Fact Sheet 2016-WHO).

B. pertussis causes 95 per cent of whooping cough cases. About 5 per cent are caused by *B. parapertussis* and infrequently, whooping cough may be caused by
B. bronchiseptica.

Laboratory Diagnosis

The bacilli are present in the upper respiratory tract most abundantly in the early stages of the disease. They may be demonstrated by microscopy or more reliably, by culture.

1. **Collection and transport of specimen** Respiratory samples can be collected by per-nasal swab, post-nasal swab (dacron or calcium alginate swabs) or using the cough plate method. The swabs are to be plated without delay or transported in a 0.25–0.5 mL Casamino acid solution, at pH 7.2, in modified Stuart's medium or Mischulow's charcoal agar.

- **Per-nasal or nasopharyngeal swab** A flexible nichrome wire is passed along the floor of the nasal cavity and material is collected from the pharyngeal wall. Nasopharyngeal aspirate collected through a soft catheter attached to a syringe is a better source. It can be used for PCR also.
- **Post-nasal (per-oral) swab** Secretions from the posterior pharyngeal wall are collected with a cotton swab on a bent wire passed through the mouth.
- **Cough plate method** A culture plate is held about 10–15 cm away from the patient's mouth during a bout of spontaneous or induced coughing so that droplets of respiratory exudates impinge directly on the medium.

2. **Microscopy** Microscopic diagnosis depends on the demonstration of the bacilli in respiratory secretions by Gram stain or fluorescent antibody technique.

3. **Cultural characteristics** It is an obligate aerobe. It grows best at 35–36°C. Complex media are necessary for primary isolation. The media commonly used are:

- **Bordet–Gengou glycerine-potato-blood agar**
- **Regan–Lowe media** with horse blood and charcoal and **cephalosporins to inhibit the normal upper respiratory flora**
- **Charcoal blood agar**—charcoal or ion exchange resins incorporated in culture media neutralise the inhibitory substances in the respiratory specimen; **charcoal**, along with starch, neutralises fatty acids and peroxides, which are toxic to *Bordetella*

Plates are incubated in high humidity at 35–36°C. Growth is slow. After incubation for 48–72 hours, colonies are small, dome-shaped, smooth, opaque, viscid, greyish-white, refractile and glistening, resembling '**bisected pearls**' or '**mercury drops**'. Confluent growth presents an '**aluminium paint**' appearance. Bacterial growth can be confirmed by direct immunofluorescence using specific antisera or biochemical tests.

4. **Biochemical reactions** *B. pertussis* is biochemically inactive. It produces oxidase and catalase.

5. **Polymerase chain reaction (PCR)** PCR-based tests are more sensitive and more commonly used now as the culture yield is poor in pertussis.

6. **Serology** **Serological diagnosis is not helpful and is not used routinely for diagnosis**.

7. **Other laboratory parameters** Marked leucocytosis is found with relative lymphocytosis (total leucocytic counts 20,000–30,000 per mm^3 with 60–80 per cent lymphocytes).

Treatment

B. pertussis is susceptible to several antibiotics (but **not penicillin**). Antimicrobial therapy is beneficial only if initiated within the first ten days of the disease. **Erythromycin or clarithromycin and azithromycin are the drugs of choice**.

Prophylaxis

1. ***Whole-cell killed vaccine*** Specific immunisation with killed *B. pertussis* vaccine has been found very effective. **Smooth phase I**

strain is used for vaccine production. Pertussis vaccine is usually administered in combination with diphtheria and tetanus toxoid (triple vaccine). *B. pertussis* also acts as an adjuvant for the toxoids, producing better antibody response.

Children under four years who are contacts of patients should receive a booster even if they had been previously immunised. They should also be given prophylaxis, preferably erythromycin. Non-immunised contacts should receive erythromycin prophylaxis for 10 days after contact with the patient has ceased.

Complications Factors contributing to toxicity or post-vaccinal encephalopathy have not been defined. Another adverse effect that can occur 48 hours after pertussis or DPT vaccination is the **hypotonic-hyporesponsive** episode (**HHE**). This is a recognised serious reaction to immunisation, especially *pertussis*-containing *vaccines*. There is decreased sensory awareness or loss of consciousness accompanied by pallor and muscle hypotonicity. *This is a reversible adverse event*. Patients recover with supportive management.

Contraindications If severe complications such as encephalopathy (1 in 5–10 million vaccinees), seizures, shock or hyperpyrexia develop following pertussis vaccination, subsequent doses of the vaccine are contraindicated. **Routine pertussis vaccination is not advisable after the age of seven years as adverse reactions are likely.**

2. ***Acellular vaccine*** Acellular vaccines containing the protective components of the pertussis bacillus (**PT, FHA, agglutinogens 1, 2, 3 and pertactin**), first developed in Japan, are now used in most countries as they cause far fewer reactions, particularly in older children.

Both whole-cell and acellular vaccines have a protection rate of about 90 per cent. With whole-cell vaccines, the protection declines to 50 per cent in about five years and is absent after 12 years. Even fully immunised subjects may develop pertussis but the disease is mild.

BORDETELLA PARAPERTUSSIS

This is an infrequent cause of whooping cough. The disease is mild. The pertussis vaccine does not protect against *B. parapertussis* infection.

BORDETELLA BRONCHISEPTICA

This is motile by peritrichate flagella. It is antigenically related to *B. pertussis* and *Brucella abortus*. It has been found to cause a very small proportion (0.1 per cent) of cases of whooping cough.

Key points

- The genus *Brucella* comprises non-motile, obligatorily aerobic, gram-negative coccobacilli that grow poorly on ordinary media and have little or no fermentative properties; the bacteria are positive in the oxidase, catalase and urease tests.
- Brucellosis is a zoonotic infection caused by a species of *Brucella*. *Brucella abortus* produces a less severe form of the disease with fewer sequelae than that caused by *Brucella suis* or *Brucella melitensis*.
- The reticuloendothelial system is affected in brucellosis. The T_h1 cell response and cell-mediated immunity are required to eliminate brucella.
- Blood, bone marrow aspirate or biopsy material are obtained for culture. Serological tests are the mainstay of diagnosis.
- There are currently no licenced vaccines for human use against brucellosis. Animal vaccines are considered unsuitable for humans.
- Members of the genus *Bordetella* are gram-negative, rod-shaped bacteria which are aerobes, non-motile and catalase-positive. *Bordetella pertussis* is by far the most important species.
- *B. pertussis* causes whooping cough. It attaches to the nasopharynx, and then grows and spreads to the ciliated cells of the bronchial tree. The bacterium secretes several toxins along with adhesins that lead to cell damage and accumulation of fluid, which induces the paroxysmal cough.
- For diagnosis, nasopharyngeal swabs or aspirates are collected. Bacteria can be cultured on charcoal agar or Bordet–Gengou medium to yield characteristic bisected pearl colonies.
- Erythromycin is given to treat active cases. Vaccination of infants and children is done as part of routine childhood immunisation, and the bacterium forms a component of the ‘triple antigen’ (diphtheria–pertussis–tetanus).
- In recent years, acellular vaccines have also become available. Aggressive vaccination to achieve herd immunity has resulted in a fall in the incidence of the disease.

Questions

Essays

1. Describe the pathogenesis and laboratory diagnosis of brucellosis.
2. Write in detail the pathogenesis, virulence factors and prevention of pertussis in children.

Short Answers

1. Serological tests for the diagnosis of brucellosis
2. Epidemiology of brucellosis
3. Milk ring test
4. Virulence factors of *B. pertussis*
5. Pertussis vaccine

Part III: Bacteriology

Chapter 35

Spirochetes

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- Treponema
  - Ultrastructure and antigens
  - Pathogenesis
  - Syphilis
  - Non-venereal syphilis
  - Congenital syphilis
  - Non-venereal treponematoses
- Borrelia
  - Relapsing fever
  - Vincent's angina
  - Lyme disease
- Leptospira
  - Classification
  - Weil's disease

INTRODUCTION

Spirochetes are elongated, motile, flexible bacteria that are twisted spirally along the long axis (their name is derived from *speira*, meaning 'coil' and *chaite*, meaning 'hair'). They are structurally more complex than other bacteria (Fig. 35.1). They have endoflagella (axial filament), which are polar, wound along the helical protoplasmic cylinder and situated between the outer membrane and the cell wall.

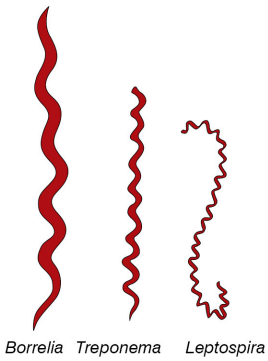


Fig. 35.1 Schematic representation of the comparative morphology of different spirochetes

Spirochetes vary widely in size, some being as long as 500 μm and others as short as 5 μm. Some of them can be stained by Gram stain and appear gram-negative (like *Borrelia,* although it is **not** classified as gram-negative bacteria). Many are free-living saprophytes, while some are obligate parasites. They may be aerobic, anaerobic or facultative. Reproduction is by transverse fission.

Among spirochetes, human pathogens are found in the genera *Treponema*, *Borrelia* and *Leptospira* (Table 35.1).

Table 35.1 Characteristics of important human spirochetes: *Treponema, Borrelia* and *Leptospira*

Properties	***Treponema***	***Borrelia***	***Leptospira***
Structure	Short, fine and tight spirals at intervals of 1 μ with tapering ends Movement is forwards and backwards	Loosely coiled with spiral morphology and presence of periplasmic flagellum	Tightly coiled spirals with hooked ends Have a corkscrew movement
Stain	Silver impregnation Giemsa staining or immunofluorescence In unstained clinical material, they can be visualised by dark-ground microscope	Giemsa Gram Appear gram-negative from human isolates May be gram-positive from tissue culture	Do not take up any stain Visualised by dark-ground microscope
Culture	Tissue culture They can be propagated in rabbit testis for use in research	Media containing serum or tissue extracts	Ellinghausen, McCullough, Johnson, Harris Medium (EMJH)

Habitat	Non-pathogenic species can be found in oral, intestinal and genital tracts	Non-pathogenic species are found in oral cavity	Soil and water Rats and other rodents
Pathogenic strains: disease spectrum	Venereal: Syphilis Non-venereal (endemic syphilis): Yaws, pinta and bejel	*B. burgdorferi*: Lyme disease *B. recurrentis*: Relapsing fever	*Leptospira interrogans*: several subspecies cause leptospirosis, Weil's disease

TREPONEMA

Treponemes *(trepos,* meaning to 'turn' and *nema,* meaning 'thread') are relatively short, slender spirochetes with fine spirals and pointed or rounded ends. Some of them are pathogenic, while others occur as commensals in the mouth, intestines and genitalia.

Treponemes cause the following diseases in humans:

- Venereal syphilis (*T. pallidum*)
- Endemic syphilis (*T. pallidum*/*T. endemicum*)
- Yaws (*T. pertenue*)
- Pinta (*T. carateum*)

They are almost identical in their morphology, antigenic structure and other features, though there are differences in the clinical features and natural history of the diseases they produce. The treponemes include *T. pallidum,* which is now considered to include three subspecies—*pallidum*, which causes venereal syphilis, *endemicum,* which causes endemic syphilis and *pertenue*, which causes yaws.

TREPONEMA PALLIDUM

Treponema pallidum, the causative agent of syphilis, was discovered by Schaudinn and Hoffmann (1905) in the chancres and inguinal lymph nodes of syphilitic patients.

Clinical Case 1

A 20-year-old male truck driver presented to a skin and venereal disease clinic with a genital ulcer of 10 days duration. The ulcer was painless. He had a history of unprotected sexual contact with a commercial

sex worker about two weeks before this. On examination, the ulcer was found to be circumscribed, indurated and partially healed (Fig. 35.2a). The inguinal lymph nodes were enlarged. A provisional diagnosis of syphilis was made and the patient's blood was sent for a VDRL test. This was reactive at 1:64 dilution. The patient was treated with intramuscular penicillin G (benzathine penicillin).

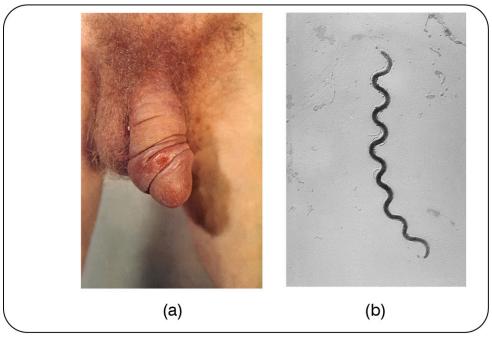


Fig. 35.2 (a) Partially healed penile ulcer, primary syphilitic lesion and (b) *Treponema pallidum* with spirals at 1 ∝ intervals seen in dark-ground microscopy (*Source:* (a) Public Health Image Library, ID 17883 / Susan Lindsley / CDC and (b) Public Health Image Library, ID 14969 / Susan Lindsley / CDC)

Morphology

T. pallidum is a thin, delicate spirochete with tapering ends, about 10 μm long (range 4–14 μm) and 0.1–0.2 μm wide. It has about ten regular spirals, which are sharp and angular, at regular intervals of about 1 μm (Fig. 35.2b). It is actively motile, exhibiting rotation around the long axis, backward and forward movements and flexion of the whole body. During motion, secondary curves appear and disappear in succession but the primary spirals are unchanged.

***T. pallidum* cannot be seen under the light microscope** in wet films. Its morphology and motility can be seen under the dark-ground or phase-contrast microscope. It does not take ordinary bacterial stains but stains a light rose-red with prolonged Giemsa staining. It can also be stained by **silver impregnation** methods. **Fontana's method** is useful for staining films and **Levaditi's method** for tissue sections.

Ultrastructure

The cytoplasm of *T. pallidum* is surrounded by a trilaminar cytoplasmic membrane, enclosed by a peptidoglycan cell wall, which gives the cell rigidity and shape. External to this, it has a lipid-rich outer membrane layer

(Fig. 35.3). Originating from each end of the cell, three or four endoflagella wind around the axis of the cell in the space between the cell wall and the outer membrane layer, to interdigitate at its centre. Endoflagella do not protrude outside but remain within the outer membrane layer.

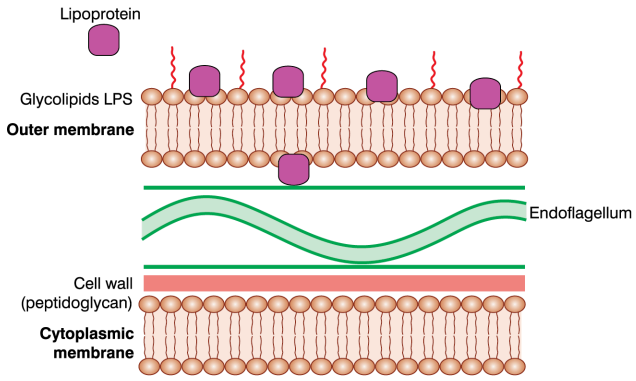


Fig. 35.3 Ultrastructure of the spirochetes showing cell wall made up of glycolipids (with LPS), peptidoglycan; the flagellum lies in the periplasmic space coiled around the axis of body of the bacterium

Antigens

The antigenic structure of *T. pallidum* is complex. There are species-specific and group-specific antigens that evoke antibody response in an infected individual.

1. **Species-specific antigen** is probably polysaccharide in nature. The antibody to this antigen is demonstrated by specific *T. pallidum* tests.
2. **Group-specific antigens** are found in *T. pallidum* as well as in non-pathogenic cultivable treponemes like the Reiter treponeme.
3. **Non-specific antigen, cardiolipin antigen,** has been detected in *T. pallidum*. **Reagin antibody** reacts with the standard or non-specific **cardiolipin antigen** in VDRL tests, in which a hapten, diphosphatidylglycerol, extracted from beef heart is used as the antigen. It is not known whether the reagin antibody is induced by the cardiolipin that is present in the spirochete or released from damaged host tissues.

(Wassermann and Kahn tests based on the same antigen are no longer used in clinical laboratories.)

Resistance

T. pallidum is readily inactivated by drying or heat (41–42°C in one hour). Fomites cannot transmit infection. The organism is killed in 1–3 days at 0–4°C. Storing blood for at least four days in the refrigerator before transfusion can prevent transfusion-associated infection. Stored frozen at –70°C in 10% glycerol or liquid nitrogen (–130°C), it remains viable for 10–15 years. It is **inactivated by distilled water, soap, arsenicals, mercurials, bismuth, common antiseptic agents and antibiotics.**

Pathogenicity

Natural infection with *T. pallidum* occurs only in human beings.

A disease resembling syphilis can be produced experimentally in chimpanzees, with typical lesions of primary and secondary syphilis. Rabbits can be infected by intradermal or intratesticular inoculation, the former giving rise to chancre and the latter to granulomatous lesions 'syphilomas'. Hamsters are also susceptible.

Syphilis

Syphilis can be:

- Venereal
- Non-venereal
- Congenital

1. Venereal syphilis

Venereal syphilis is acquired by sexual contact. The spirochete enters the body through minute abrasions on the mucosa or skin. Individuals with primary, secondary and early latent stages of syphilis can transmit the infection.

Treponema multiplies at the site of entry. Its generation time is 30–33 hours. Clinical disease sets in after an **incubation period of about a month** (range 10–90 days). The clinical manifestations fall into **four stages**: primary, secondary, latent and tertiary.

i) **Primary syphilis** is characterised by the chancre at the site of entry of the spirochete, usually the genitalia (Fig. 35.4). The chancre is a painless, relatively avascular, circumscribed, indurated and

superficially ulcerated lesion (*Clinical case 1*). It is known as 'hard chancre' to distinguish it from the non-indurated lesions of 'soft chancre' caused by *H. ducreyi*. The chancre is covered by a thick, glairy exudate rich in spirochetes.

Fig. 35.4 Indurated penile ulcer (chancre) in primary syphilis (*Source:* Public Health Image Library, ID 6803 / M.Rein / CDC)

Other common sites are the mouth and nipples.

- The regional lymph nodes are swollen, discrete, rubbery and non-tender.
- Spirochetes spread from the site of entry into the lymph and bloodstream even before the chancre appears, so the patient may be infectious during the late incubation period.
- The chancre invariably heals in about 10–40 days, even without treatment, leaving a thin scar.

ii) **Secondary syphilis** sets in 1–3 months after the primary lesion heals. During this interval, the patient is asymptomatic.

- The secondary lesions are due to the multiplication of the spirochetes and their dissemination through blood.

Condylomata at the mucocutaneous junctions are the characteristic lesions (Fig. 35.5a). Roseolar or papular skin rashes, mucous patches in the oropharynx and papulosquamous lesions on the palms and feet may (which may be confused as psoriatic lesions) are typical features of secondary syphilis (Fig. 35.5b and c).

(a) (b) (c)

Fig. 35.5 Secondary syphilis: (a) condylomata at the vulvovaginal mucocutaneous junction in a female patient; (b) papulosquamous rashes on the palm in secondary syphilis and (c) maculopapular rash on the back in secondary syphilis (*Source:* (a and b) Dr. Sheela Kuruvila, Department of Dermatology, Pondicherry Institute of Medical Sciences, Puducherry and
(c) Public Health Image Library, ID 17838 / Dr. Gavin Hart / CDC)

- Spirochetes are abundant in the lesions and consequently, the patient is most infectious during the secondary stage.
- There may also be ophthalmic, osseous and meningeal involvement.
- Secondary lesions are highly variable in distribution, intensity and duration but they usually undergo spontaneous healing, in some instances, taking as long as four or five years.

iii) **Latent syphilis** is seen after the secondary lesions disappear and there is a period of quiescence. Diagnosis during this period is possible only by serological tests. In many cases, this is followed by natural cure.

iv) **Tertiary syphilis** appears, in some, after several years. The disease manifests as:

- Cardiovascular lesions including aneurysms and chronic granulomata (gummata) (Fig. 35.6)
- Meningovascular manifestations
- Tertiary lesions containing few spirochetes; may represent manifestations of delayed hypersensitivity

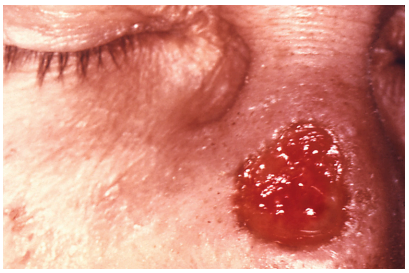


Fig. 35.6 Tertiary syphilitic lesion showing a cutaneous ulcerative lesion on the nose (*Source:* Public Health Image Library, ID 5330 / J. Pledger / CDC)

Late tertiary In a few cases, a late tertiary stage may develop, presenting with neurological manifestations such as:

- Tabes dorsalis
- General paralysis of the insane (GPI)

These may develop several decades after the initial infection.

2. Non-venereal syphilis

Syphilis can be acquired non-venereally (it is an occupational hazard for doctors or nurses). The natural evolution is as in venereal syphilis, except that the primary chancre is extragenital, usually on the fingers. If syphilis is transmitted by blood transfusion, the primary chancre does not develop.

3. Congenital syphilis

The infection is transmitted from an infected mother to her fetus transplacentally. Transplacental transmission can take place at any stage of the pregnancy. Infected infants may be asymptomatic or may have subtle findings or multiple-organ involvement. Even asymptomatic newborns may have early or late postnatal manifestations.

The lesions of congenital syphilis usually develop only after the fourth month of gestation. Pathogenesis is the result of an immune response from the fetus. In untreated women, history of repeated abortions is suggestive of congenital syphilis. Congenital syphilis can manifest late in children born to infected mothers. Late congenital syphilis may involve the bones, resulting in osteoperiostitis (Fig. 35.7).

Fig. 35.7 Late congenital syphilis involving the bones—osteoperiostitis and ulnar deviation of the middle finger (*Source:* Public Health Image Library, ID 16750 / Renelle Woodall / CDC)

Epidemiology

Syphilis is transmitted sexually and is worldwide in distribution. It was hoped that the spread of the disease could be contained with penicillin treatment; however, this has not been possible.

HIV and syphilis The advent of the AIDS pandemic has had an impact on syphilis. In most places, the fear of AIDS and safer sex practices led to a drop in the incidence of syphilis and all STDs. Concurrent infection with syphilis and HIV is common and may lead to earlier evolution of neurosyphilis. Detection, diagnosis and treatment of concurrent infections of HIV and syphilis and other sexually transmitted infections (STIs) have been included in the control programme strategy.

Immunity

The immune mechanisms in syphilis are not adequately understood. Humoral immune response against the treponeme does not appear to be effective as the disease progresses even in the presence of a vigorous antibody response. Specifically sensitised T_h1 cells secrete cytokines that may favour the clearance of spirochetes by activated macrophages.

Re-infections do not appear to occur in a person with an active infection.

Laboratory Diagnosis

Laboratory diagnosis consists of the demonstration of the spirochetes under the microscope and of antibodies in serum or CSF. Culture does not have a role in diagnosis.

1. **Specimen** Specimens from the genital ulcer should be collected with care as the lesions are highly infectious. Blood is collected for serology. CSF can

be collected for serology in the tertiary stage to confirm neurosyphilis.

2. **Microscopy**

- **Dark-ground microscopy** can be used for the demonstration of treponemes in primary and secondary stages and in congenital syphilis with superficial lesions. Wet films are examined under the dark-ground microscope. *T. pallidum* is identified by its slender spiral structure, slow backward and forward movements and bending at right angles.

  Dark-ground microscopy is useful as it can be done at the outpatient or point-of-care outlet. However, it has low sensitivity. A treponemal concentration of 104 per mL in the exudates is required to visualise the bacteria (Fig. 35.8a). Due to low sensitivity, several specimens have to be screened over consecutive days.
- Direct fluorescent antibody test for *T. pallidum* (DFA-TP) is a better and safer method for microscopic diagnosis. Smears of the exudate are fixed with acetone. DFA-TP test is done using fluorescent-tagged monoclonal anti-*T. pallidum* antiserum.
- Silver impregnation methods include Fontana's method, which is useful for staining films and Levaditi's method for tissue sections (Fig. 35.8b).

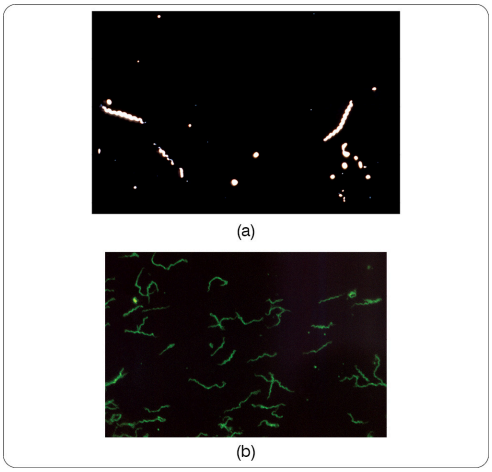


Fig. 35.8 (a) Dark-field microscopy shows closely spiralled *Treponema pallidum* and (b) silver impregnation method of Levaditi to demonstrate spirochetes in tissue (*Source:* (a) Public Health Image Library, ID 14968 / Renelle Woodall / CDC)

(b) Public Health Image Library, ID 14967 / Russell / CDC)

3. **Cultivation** This is **not** used in laboratory confirmation of syphilis. Pathogenic treponemes do not grow in artificial culture media.

- Cultivable strains are the non-pathogenic treponemes, showing morphological and antigenic similarities with *T. pallidum*. Reiter strain is widely used as the antigen in group-specific treponemal tests for the diagnosis of syphilis. The Reiter treponeme grows well in thioglycolate medium containing serum. It is now named as *T. phagedenis*.
- Virulent *T. pallidum* strains have been maintained for many decades by serial passage in rabbit testis. Nichols strain, isolated from the brain of a fatal case of general paralysis of the insane in 1912, is still being propagated and used for diagnostic and research purposes.

4. **Serological tests** These tests form the mainstay of laboratory diagnosis. There are three types of tests used to detect the antibodies: (i) standard tests for syphilis, (ii) group-specific treponemal tests for syphilis and (iii) specific *T. pallidum* tests for syphilis.

i) Standard tests for syphilis (STS) utilise the cardiolipin antigen to detect the reagin antibody. The antigen is a purified lipid extract of beef heart with added lecithin and cholesterol.

> The antibody that reacts with cardiolipin is known as reagin. This can be misleading as the IgE antibody in atopy is also called reagin, though there is no connection between the two.

- **VDRL (Venereal Disease Research Laboratory, USPHS, New York, where the test was developed)** test is rapid and gives quantitative results.
  - Inactivated serum (serum heated at 56°C for 30 minutes) is mixed with cardiolipin antigen on a special slide (VDRL-slide) and rotated for four minutes. Cardiolipin remains as uniform crystals in normal serum but forms visible clumps on combining with the reagin antibody. The reaction is read under a low-power microscope.

- **Antibody titre interpretation** can be done by testing serial dilutions. The results are reported qualitatively as 'reactive', 'weakly reactive' or 'not reactive'. For quantitative reporting, the reciprocal of the end point is given as the titre, for example, 'reactive 4 dilution' or 'titre 4'.
- The VDRL test can be used for testing CSF in neurosyphilis also, but not in the plasma of these patients. CSF need not be heated prior to the test.

- **Rapid plasma reagin** (RPR) is the most popular of the modified VDRL tests developed. This test uses the VDRL antigen containing fine carbon particles, which make the result more clear-cut and evident to the naked eye. The RPR test can be done with unheated serum or plasma but is not suitable for testing CSF. **Automated RPR test** (ART) is available for rapid detection in a large number of samples.
- **Toluidine red unheated serum test** (TRUST) is similar to RPR. It is a rapid test done on a card. CSF can also be tested, and the results are similar to those of VDRL.
- **Unheated serum reagin test** (USRT) is a microscopic flocculation test to detect anti-cardiolipin antibodies. It is also a non-treponemal screening test to diagnose and monitor treatment.

Disadvantages of STS

Biological false positive (BFP) reactions As the cardiolipin antigen is a non-specific antigen, biological false positive (BFP) reactions are seen due to unconnected infections. This is a major disadvantage of STS.

- BFP reactions may occur in about one per cent of normal sera. BFP antibody is usually IgM, while reagin antibody in syphilis is mainly IgG.
- Acute BFP reactions last only for a few weeks or months and are usually associated with acute infections, injuries or inflammatory conditions.
- Chronic BFP reactions persist for longer than six months and are typically seen in SLE and other collagen diseases. Leprosy, malaria, relapsing fever, infectious mononucleosis, hepatitis and tropical eosinophilia are examples of other conditions associated with BFP reactions.
- The reagin antibody becomes detectable 7–10 days after the appearance of primary chancre (or 3–5 weeks after acquiring the infection). Sensitivity in the primary stage is 60–75 per cent. In the secondary stage, sensitivity is 100 per cent and titres range from 16 to 128 or more.

- Non-treponemal serological tests may be false negative in very early and late syphilis or due to prozone phenomenon. Hence when there is strong clinical suspicion, serum must have high dilutions to detect prozone.

Congenital syphilis results in high titres of antibodies. After the secondary stage, titres diminish and about a third of patients with late syphilis are seronegative. **The titres may rise in patients developing cardiovascular, neurological or gummatous lesions**. In some cases of neurosyphilis, reagin tests may be negative with serum but positive with CSF. **STS becomes negative 6–18 months after effective treatment of syphilis**, depending on the stage at which treatment is given. However, if treatment is started late, the tests may remain positive in low titres.

ii) Group-specific treponemal tests These depend on the use of Reiter's protein as the antigen tested by complement fixation test. However, these tests are obsolete now.

iii) **Specific *T. pallidum* tests** These tests use the virulent Nichols strain of *T. pallidum* maintained by serial inoculation in rabbit testes.

- **Tests using strains of *T. pallidum***
  - **Fluorescent treponemal antibody (FTA)** test is an indirect immunofluorescence test using as antigen, smears prepared on slides with Nichols strain of *T. pallidum.*
  - **FTA-absorption (FTA-ABS)** is the currently used modification of FTA test. The test serum is pre-absorbed with a sonicate of the Reiter treponemes (**sorbent**) to eliminate group-specific reactions. FTA-ABS is as specific as the ***Treponema pallidum* immobilisation** test and is now accepted as a standard reference test. However, as it can be done only in suitably equipped laboratories, it is not available for routine testing.
  - ***Treponema pallidum* immobilisation (TPI)**uses test serum incubated with a suspension of complement and *T. pallidum* (maintained in a complex medium anaerobically). If antibodies are present, the treponemes are immobilised. This can be viewed under dark-ground illumination. However, because of its extreme complexity, it is not preferred. The TPI

test has now been replaced by others such as FTA-ABS and TPHA, which are as specific and much simpler to perform.

- **Tests using extracts of *T. pallidum***
  - ***T. pallidum* hemagglutination assay** (TPHA) uses tanned erythrocytes sensitised with a sonicated extract of *T. pallidum* as the antigen (Fig. 35.9). The procedure in use is a micro-hemagglutination test (MHA-TP), which can be automated. The test sera for TPHA are absorbed with a diluent containing components of the Reiter treponeme, rabbit testis and sheep erythrocytes. Sera are screened at an initial dilution of 1:80 but titres as high as 1:5,120 or more are common in the secondary stage. TPHA is as specific as FTA-ABS and almost as sensitive, except in the primary stage. Kits are available commercially. These advantages have made TPHA a standard confirmatory test.

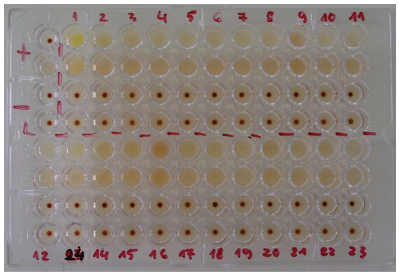


Fig. 35.9 *T. pallidum* hemagglutination assay (TPHA) on a microtitre plate; tanned erythrocytes sensitised with sonicated extracts of *T. pallidum* added to dilutions of the patient's serum showing agglutination (positive) and buttons showing negative titres

- TPPA—*Treponema pallidum* particle agglutination test is a passive agglutination test, similar in principle to TPHA. Sonicated *T. pallidum* (Nichols strain) antigen is coated to gelatin particles. These agglutinate with antibodies to *T. pallidum* found in the patient's serum.

Table 35.2 shows the relative sensitivities of the serological tests in common use.

Table 35.2 Frequency of reactive serological tests in untreated syphilis (percentage)

Stage	VDRL/RPR	FTA-ABS	TPHA
Primary	70–80	85–100	65–85

Secondary	100	100	100
Latent/late	60–70	95–100	95–100

- Enzyme immunoassays (EIA) have been developed using *T. pallidum* antigens and are available commercially (Bio-Enza Bead test; Captia Syphilis-G test). A rapid agglutination test (TPPA) has been developed, using gelatin or latex particles coated with three immunodominant proteins of *T. pallidum*, obtained by recombinant technology. It is claimed to be as specific as TPHA and more sensitive.
- Molecular methods, both conventional and real-time PCR, have been used to detect *T. pallidum* DNA from tissues and other clinical material in syphilis. They can detect DNA at all the stages and in a short time. This is used as a rapid diagnostic tool in the absence of direct immunofluorescence or dark-ground microscopy.

Laboratory Diagnosis of Congenital Syphilis (CS)

The diagnosis of congenital syphilis is complex and is based on a combination of laboratory criteria in both mother and infant. Often, there is an absence of specific signs of infection at birth; hence serology has a crucial role in the diagnosis of congenital syphilis.

As IgM does not cross the placenta, its presence in neonatal serum confirms congenital syphilis and helps differentiate it from seropositivity due to passively transferred maternal antibody. IgM antibody titres in the infant at the time of delivery and in the mother are detected using non-treponemal tests (VDRL, RPR)—a serology titre of IgM in the infant that is equal to or four times that in the mother is strongly suggestive of congenital infection. However, the absence of a ≥ 4-fold titre does not rule out congenital infection. Selective detection of IgM antibodies by modifications of the FTA-ABS, TPHA, EIA and VDRL tests using whole sera or separated IgM fractions have been developed.

Western blot This is an immunoblot technique which uses whole-cell antigen or recombinant polypeptide antigens of *T. pallidum* on

nitrocellulose. The test can detect IgM and IgG in congenital syphilis. It is a useful confirmatory test. Besides congenital syphilis, western blot is also useful in confirming syphilis in patients with SLE and other autoimmune diseases.

Treponema pallidum-specific DNA detection by PCR may help to diagnose congenital neurosyphilis. However, CSF tests for VDRL and RPR are equally effective and less costly.

According to the testing guideline for syphilis screening (WHO STI guidelines), all pregnant women must be screened for syphilis in the first antenatal care visit.

Algorithm for testing

- **Screening for syphilis** This is done first by standard tests (VDRL or RPR). The main advantage of these tests is their low cost and availability in point-of-care laboratories.
- **Response to treatment** Quantitative tests are useful in monitoring the patient's response to treatment, indicating the stage of the disease and in detecting re-infection. VDRL or RPR are preferred because they usually become negative following treatment.
- A reverse algorithm is used in some countries where the first step is to test sera for *Treponema pallidum*-specific IgG antibodies using immunoassay. However, IgG antibodies to syphilis can remain elevated despite appropriate antimicrobial treatment and a reactive result does not distinguish between recent or past infection. To further evaluate disease and treatment status, samples are subsequently tested by rapid plasma reagin (RPR) assay or VDRL, which detects anticardiolipin antibodies. This helps to monitor response to treatment. If treatment is given very early, the serum may not become positive at all. Treatment in the primary stage leads to seroreversal in about four months; in the secondary and early latent stages, it takes 12–18 months; in later stages, it may take five years or more.

Diagnostic accuracy **TPHA** and **FTA-ABS** are helpful in excluding or confirming the diagnosis of syphilis. Both TPHA and FTA-ABS can give false positive results, though very rarely. All serological tests for syphilis may be positive in non-venereal treponematoses, and some in a few other spirochetal infections as well. In Lyme disease, the VDRL test is negative but FTA-ABS may be positive (Table 35.3).

Table 35.3 Reactivity of serological tests by stage of syphilis and effect of treatment

Serological tests	Antigen used	Antibodies detected	Implications
Screening tests Standard tests for syphilis (STS)	Cardiolipin	IgG/IgM	**Positive** Early infection (primary and secondary syphilis)

VDRL RPR			Tertiary: CSF in neurosyphilis **Negative** Tertiary and latent Successful treatment Non-venereal syphilis
Confirmatory tests: specific treponemal antigen-based: FTA-ABS, TPHA, TPPA, EIA	*T. pallidum*: Reiter's or Nichols strains	IgG/IgM	**Positive** All stages of syphilis Remains positive despite treatment May be positive in Lyme disease **Negative** Rules out syphilis
Western blot	Whole-cell lysate of *T. pallidum* Or recombinant polypeptide antigens of *T. pallidum*	IgG or IgM	Positive in infant's serum in congenital syphilis Syphilis in patients with SLE and other autoimmune diseases

A **negative TPHA** virtually excludes the diagnosis of syphilis, past or present, except in the very early stages. In neurosyphilis, a negative CSF VDRL test may not be conclusive but a negative TPHA test eliminates the possibility of neurosyphilis. Detection of **specific IgM antibody** may be helpful in some situations.

A summary of the serological tests of syphilis by stage and the effects of treatment are shown in Fig. 35.10.

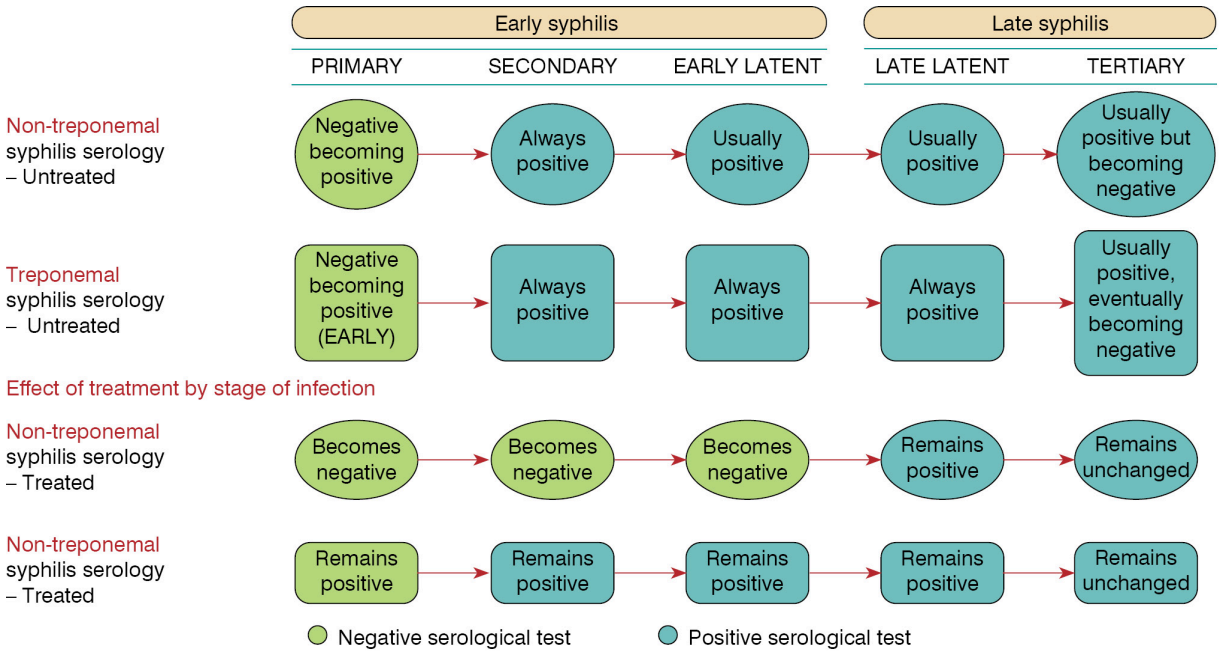


Fig. 35.10 Reactivity of serological tests by stage of syphilis and effect of treatment (*Source:* WHO Guideline on Syphilis Screening and Treatment for Pregnant Women)

Prophylaxis

As transmission is by direct contact, syphilis can be prevented by avoiding sexual contact with an infected individual. The use of physical barriers (such as condoms), antiseptics (potassium permanganate) or antibiotics may minimise the risk. The use of prophylactic penicillin carries a possible danger of suppressing the primary lesion without eliminating the infection, as a result of which, recognition and treatment of the disease may become more difficult. No vaccine is available.

Treatment

Penicillin is uniformly effective in syphilis but it is necessary to give an adequate dose and maintain the drug level for a sufficiently long period to establish cure.

- **Early syphilis** A single injection of 2.4 million units of benzathine penicillin G is adequate.
- **Late syphilis** The above dose may be repeated weekly for three weeks. In patients allergic to penicillin, doxycycline may be used. Ceftriaxone is effective, particularly in neurosyphilis.

Penicillin treatment in syphilis sometimes induces the **Jarisch–Herxheimer reaction**, consisting of fever, malaise and exacerbation of symptoms. It is frequent but harmless in primary and secondary syphilis and can be managed with bed rest and aspirin.

Monitoring after treatment

All patients must be counselled and treated along with their partners. As the course is long, they must be **monitored** to ensure compliance with treatment. Patients with HIV infection and patients who are on non-penicillin regimen should be screened and tested at 3, 6, 9 and 12 months. Patients with latent syphilis should be monitored with RPR or VDRL at 6-, 12- and 24-month intervals. An integrated approach to diagnosis, treatment and monitoring of sexually transmitted infections (STI) and HIV and TB-HIV is underway in the national programme.

Non-venereal Treponematoses

Non-venereal treponemal diseases occur in endemic foci in several parts of the world, among communities with poor standards of hygiene. Three distinct forms of non-venereal treponematoses are recognised—(i) bejel/siti or endemic syphilis, (ii) yaws and (iii) pinta (Table 35.4).

Table 35.4 Comparative characteristics of venereal and non-venereal syphilis

	Venereal syphilis	Non-venereal syphilis
Causative agent	*T. pallidum*	*T. pallidum* subsp. *endemicum* (bejel) *T. pallidum* subsp. *pertenue* (yaws) *T. carateum* (pinta)
Transmission	Sexual contact Congenital Non-sexual—direct contact (doctors/nurses)	Direct contact No congenital transmission reported
Laboratory diagnosis	Serological Standard tests *Treponema*-specific tests	Same as in syphilis due to antigenic similarity
Geographic location	Worldwide	Specific geographic endemicity

Endemic syphilis

Syphilis, transmitted non-venereally, was endemic in several foci. It is

known as bejel in Saudi Arabia and in the Balkans (nomadic tribes) or siti in Gambia. It has also been reported from India. The causative agent is *T. pallidum* subspecies *endemicum*. With the recognition of such foci and mass treatment with penicillin under the auspices of the WHO, endemic syphilis has become very rare.

The disease is common in young children. The primary chancre is not usually seen, except sometimes on the nipples of mothers infected by their children. The disease is usually seen with manifestations of secondary syphilis, such as mucous patches and skin eruptions. The disease progresses to tertiary lesions, particularly gummatous lesions. Cardiovascular and neurological involvement is rare. Congenital syphilis is also not found. Laboratory diagnosis and treatment are as for venereal syphilis.

Yaws

Yaws, also known as frambesia, pian and parangi, is endemic in the tropical areas of Africa, Asia and America. Yaws eradication campaigns by mass penicillin injection in endemic areas led to the virtual eradication of the disease. In India, cases have been identified in Andhra Pradesh, Odisha and Madhya Pradesh.

The causative agent is a *T. pallidum* subspecies, pertenue (*T. pertenue*), which is morphologically and antigenically indistinguishable from *T. pallidum*. The primary lesion (mother yaw) is an extragenital papule which enlarges and breaks down to form an ulcerating granuloma (Fig. 35.11). As in syphilis, secondary and tertiary manifestations follow, but cardiovascular or neurological involvement is rare. Destructive gummatous lesions of the bones are common.

Fig. 35.11 Yaws: ulcerative granuloma (*Source:* Public Health Image Library, ID 3841 / Dr. Peter Perine / CDC)

Pinta

Pinta (also known as azur, carate and mal del pintois) is endemic in Mexico, Central and South America and the neighbouring islands. The primary lesion is an extragenital papule, which does not ulcerate but develops into a lichenoid or psoriasiform patch. Secondary skin lesions are characterised by hyperpigmentation or hypopigmentation (Fig. 35.12). Tissues other than skin are seldom affected. The causative agent is *T. carateum*. It is very closely related to *T. pallidum* but is not antigenically identical, so cross-immunity between pinta and syphilis is only partial.

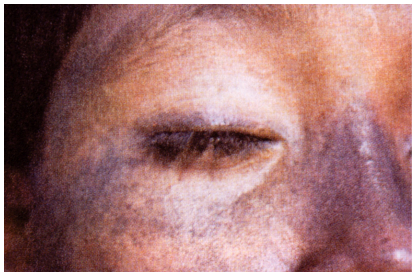


Fig. 35.12 Discoloured areas of facial skin in pinta (*Source:* Public Health Image Library, ID 15696 / Dr. J.G. Caldwell / CDC)

Treatment

Benzathine penicillin or oral dose of azithromycin have been recommended for treatment.

NON-PATHOGENIC TREPONEMES

Several commensal treponemes (e.g., *T. denticola*) occur on the buccal and genital mucosa and may cause confusion in the diagnosis of syphilis by dark-field examination. They are a heterogeneous group and have not been adequately characterised.

BORRELIA

Borreliae are large, motile, refractile spirochetes with irregular, wide and open coils. They are usually 5–30 μm long and 0.3–0.7 μm wide. They are readily stained by ordinary methods and are gram-negative. Several species of *Borrelia* occur as commensals on the buccal and genital mucosa. Borreliae of medical importance are *B. recurrentis,* which causes relapsing

fever, *B. vincentii,* which causes Vincent's angina and *B. burgdorferi,* which causes Lyme disease.

BORRELIA RECURRENTIS (REPLAPSING FEVER)

Borrelia recurrentis causes **relapsing fever (RF)**, an arthropod-borne infection, two types of which occur: louse-borne and tick-borne.

i) Epidemic or louse-borne RF This is the type of RF caused by *B. recurrentis*. It is an exclusively human pathogen and is transmitted from person to person through body lice (*Pediculus humanus corporis*). No extra-human reservoir is known.

ii) Endemic or tick-borne RF This is caused by *B. duttonii, B. hermsii, B. parkeri,* etc. Such infections are generally confined to certain geographic areas. Borreliae that cause endemic RF normally live in their natural hosts—rodents or other mammals on which the vector ticks feed. Human infection is only an accidental event.

DNA homology studies indicate that all of them may belong to a single species, with separate host adaptation. The descriptions that follow apply to all of them, unless stated otherwise.

Morphology

B. recurrentis is an irregular spiral with one or both ends pointed. It possesses 5–10 loose spiral coils at intervals of about 2 μm. It stains well with Giemsa and bacterial stains and is gram-negative.

Antigenic Properties

Borrelia readily undergoes antigenic variations in vivo and this is believed to be the reason for the occurrence of relapses in the disease.

Pathogenicity

After an incubation period of 2–10 days, relapsing fever sets in as fever of sudden onset. During this period, borreliae are abundant in the patient's blood. The fever subsides in 3–5 days. After an afebrile period of 4–10 days, during which borreliae are not demonstrable in blood, another bout of fever sets in. The borreliae reappear in blood during the relapses of fever. The disease ultimately subsides after 3–10 relapses.

Epidemiology

Epidemic or louse-borne relapsing fever tends to occur as epidemics whenever poverty, overcrowding and lack of personal hygiene encourage louse infestation. The louse-borne disease presents a more severe clinical picture than the tick-borne variety and is associated with jaundice, hemorrhages and, in some outbreaks, a high rate of fatality. In lice, the *Borrelia* is confined to the hemolymph and is not shed in saliva or excreta. So the infection is transmitted not by the bite of lice but by their being crushed and rubbed into abraded skin. *B. recurrentis* is not transmitted transovarially in lice.

Endemic or tick-borne relapsing fever occurs as sporadic cases in endemic areas. The *Borrelia* persists in the body of infected ticks throughout their life and is also transmitted transovarially so that the ticks act as reservoirs as well as vectors. The infection is transmitted to humans through the bite of ticks or their discharges. **In India, the vector species are *Ornithodoros tholozoni, O. crossi, O. lahorensis* and the fowl tick, *Argas persicus***. Relapsing fever may very rarely be acquired congenitally by transplacental transfer. Laboratory infection may occur through contact with the blood of patients or experimental animals.

Laboratory Diagnosis

1. **Microscopic examination** Borreliae are found in the blood during the fever but seldom in the apyrexial intervals.

Wet preparation A drop of blood may be examined as a wet film under the dark-ground or phase-contrast microscope and borreliae detected by their lashing movement.

Staining Blood smears may be stained with Giemsa or Leishman stain or with dilute carbol fuchsin and examined for borreliae.

Quantitative buffy coat is more sensitive than thick films and may be useful in the diagnosis of relapsing fever.

2. **Animal inoculation** A more successful method is to inoculate 1–2 mL of blood from the patient intraperitoneally into white mice. Blood smears from the mice become positive within two days.

3. **Culture** *Borrelia* are microaerophilic. Optimum temperature for growth

is 28–30°C. Cultivation is difficult but has been successful in complex media containing serous fluids and in the chorioallantoic membrane of chick embryos. Routine cultivation of borreliae for diagnosis is not very successful and hence not routinely used in diagnosis.

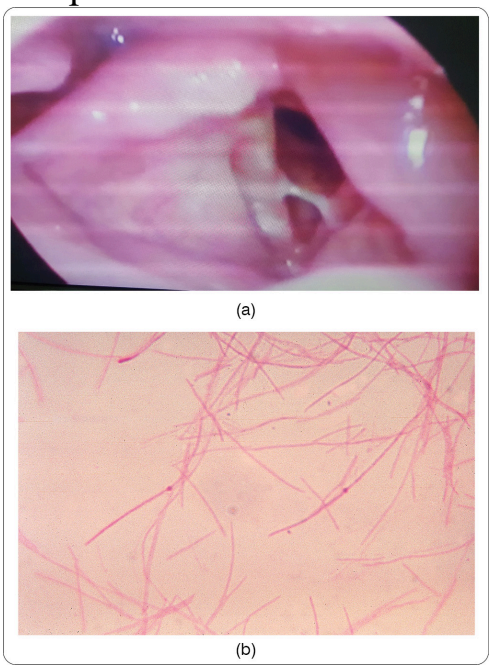
4. **Serology** Whole-cell lysates of the spirochete are used as antigens for ELISA and IFA for the diagnosis of relapsing fever. Agglutinins for *Proteus* OXK are sometimes seen in high titres in louse-borne relapsing fever.

Treatment

Tetracyclines, chloramphenicol, penicillin and erythromycin are effective. No vaccine is available.

BORRELIA VINCENTII (VINCENT'S ANGINA)

Borrelia vincentii is a motile spirochete, about 5–20 μm long and 0.2–0.6 μm wide, with 3–8 coils of variable size. It is easily stained with dilute carbol fuchsin and is gram-negative. It is a normal mouth commensal but may, under predisposing conditions such as malnutrition or viral infections, give rise to ulcerative gingivostomatitis or oropharyngitis (Vincent's angina) (Fig. 35.13a). In these cases, *B. vincentii* is always associated with fusiform bacilli (*Fusobacterium fusiforme*) (Fig. 35.13b). This symbiotic infection is known as fusospirochetosis.


(a)

(b)

Fig. 35.13 (a) Oropharyngitis (Vincent's angina); patient recovered dramatically with penicillin and (b) fusiform bacilli from Vincent's angina (*Source:* (a) Department of ENT, Pondicherry Institute of Medical Sciences, Puducherry and (b) Public Health Image Library, ID 3044 / Dr. V.R. Dowell, Jr. / CDC)

Diagnosis may be made by demonstrating spirochetes and fusiform bacilli in stained smears of exudates from the lesions. *B. vincentii* may be anaerobically cultivated with difficulty in enriched media. Penicillin and metronidazole are effective in treatment.

BORRELIA BURGDORFERI (LYME DISEASE)

Lyme disease or **Lyme borreliosis** (originally **Lyme arthritis**) was so named as it was first observed in Lyme, Connecticut, USA. The disease is widespread in the USA, where it is the most common vector-borne infection. It has been reported from other parts of the world also. It is caused by *Borrelia burgdorferi*, which is transmitted by the bite of Ixodid ticks. It is a fastidious bacterium. Three species of *Borrelia* have been identified (*B. burgdorferi*, *B. garinii* and *B. afzelii*), each of which is prevalent in different geographical regions, causing regional variations in clinical features.

The natural reservoir hosts are rodents, deer and other mammals.

Clinical Presentation

Lyme disease occurs in **three stages**:

i) After an incubation period of 3–30 days, the first stage, localised infection, appears as an expanding annular skin lesion (erythema migrans or EM) (Fig. 35.14a).

ii) A few weeks later, the second stage, disseminated infection, develops with fever, headache, myalgia, arthralgia and lymphadenopathy. Some develop meningeal involvement or meningoradiculitis, which is a neuroinvasive syndrome (Bannwarth's syndrome), or cardiac involvement, with atrioventricular conduction defect (Fig. 35.14b).

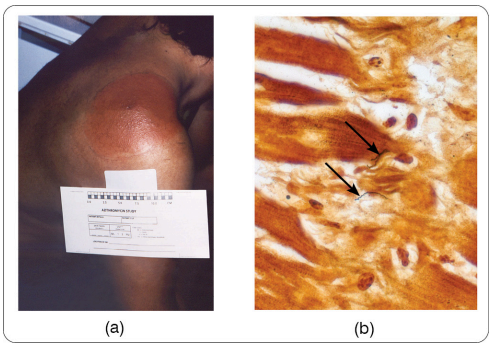


Fig. 35.14 (a) An expanding annular skin lesion (erythema migrans or EM, caused by *B. burgdorferi*) and (b) *B. burgdorferi* in the heart tissue, stained with Warthin–Starry stain (*Source:* (a) Public Health Image Library, ID 14482 / CDC and (b) Public Health Image Library, ID 14482 / Sherif Zaki, M.D. Ph.D.; DVBD / CDC)

iii. The third stage, **persistent infection**, sets in months or years later with chronic arthritis, polyneuropathy, encephalopathy and acrodermatitis.

Laboratory Diagnosis

Though borreliae have been isolated from ticks as well as from skin lesions, CSF and the blood of patients, culture is too slow and difficult to be of use in diagnosis**.**

Biopsy tissue from infected skin, synovial tissue or synovial fluid may be used to detect *Borrelia burgdorferi* DNA by **real-time** or **conventional PCR**.

Serological tests Tests such as ELISA and IF have been described and immunoblotting recommended for confirmation.

Antibodies take 1–2 months to appear, with initial IgM response followed by IgG. False positive syphilis serology may be seen with a positive FTA-ABS and a negative VDRL.

Treatment

Doxycycline, amoxiycillin and cefuroxime are useful antibiotics to treat Lyme disease.

LEPTOSPIRA

Leptospires are actively motile, delicate spirochetes, possessing a large number of closely wound spirals and characteristic hooked ends. They are

too thin to be seen under the light microscope (*leptos,* meaning fine or thin). Several leptospires are saprophytic, while many are parasitic in rodents and other animals.

Clinical Case 2

A 45-year-old woman who worked in a rice farm where she would stand in water during planting season, developed fever off and on and did not seek medical advice. After two weeks, she presented with suffused conjunctiva, jaundice and continuous fever. A diagnosis of leptospirosis was made. Her sera tested positive by ELISA for IgM antibodies to leptospira antigens. For confirmation, her serum sample was sent to the reference laboratory for microscopic agglutination test for leptospira-specific antibodies. This was positive at a titre of 1:10,000. She was treated with doxycycline.

Morphology

Leptospires are delicate, flexible, helical rods about 6–20 µm long and 0.1 µm thick. They possess numerous coils set so close together that they can be distinguished only under dark-ground illumination in the living state or by electron microscopy. Their ends are hooked and resemble umbrella handles (Fig. 35.15). They are actively motile and stain poorly with aniline dyes. They may be stained with Giemsa stain but better results are obtained by the silver impregnation methods.

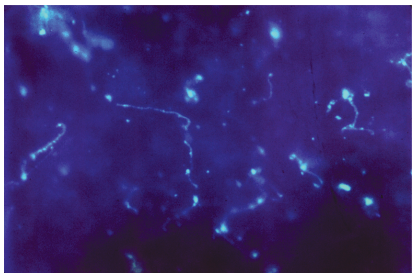


Fig. 35.15 Fluorescent antibody (FA) staining of a liver impression smear sample from a fatal case of leptospirosis shows corkscrew-shaped *Leptospira* spp. (*Source:* Public Health Image Library, ID 1346 / Mildred Galton / CDC)

Classification

The genus *Leptospira* is now classified into two species, and within each species are serogroups, which are further classified into serotypes (serovars).

1. ***L. interrogans*** contains pathogenic leptospires, with about 22 serogroups having more than 200 serotypes (Table 35.5). *L. interrogans* is classified into several serogroups (*Icterohaemorrhagiae, Canicola, Pyrogenes, Autumnalis, Australis, Pomona, Hebdomadis,*

Grippotyphosa, etc.). Further, serogroup *Icterohaemorrhagiae* contains serovars *icterohaemorrhagiae, copenhageni, smithi,* etc.).

Table 35.5 Important leptospiral infections (*L. interrogans*)

Serogroup	Disease	Clinical picture	Animal reservoir	Distribution
Icterohemorrhagiae	Weil's disease	Fever, jaundice, hemorrhages	Rat	Worldwide
Canicola	Canicola fever	Influenza-like, aseptic meningitis	Dog	Worldwide
Grippotyphosa	Swamp or marsh fever	Fever, prostration, aseptic meningitis	Field mice	Europe, Africa, SE Asia, USA
Pomona	Swineherd's disease	Fever, inflammation of the blood vessels	Pig	America, Europe, Middle East, Indonesia, Australia
Hebdomadis	Seven day fever	Fever, lymphadenopathy	Field mice	Japan, Europe, USA
Fortbragg	Pretibial fever Fort Bragg fever	Fever, rash over tibia	Not known	Japan, SE Asia, USA
Pyrogenes	Febrile spirochetosis	Fever	Pig	SE Asia, Europe, USA
Bataviae	Indonesian Weil's disease	Fever	Rat	SE Asia, Africa, Europe
Hardjo	Dairy farm fever	Fever	Cattle	UK, USA, New Zealand

2. ***L. biflexa*** contains saprophytic leptospires found predominantly in surface waters. Over 200 serovars have been identified and assembled into 22 serogroups.

Antigenic Properties

Leptospires exhibit considerable antigenic cross-reaction. A genus-specific somatic antigen is present in all members of the genus. **Classification into serogroups and serotypes is based on surface antigens**. Determination of serotypes is based on agglutination and cross-absorption reactions using immune rabbit sera or more recently, with monoclonal antibodies. Genetic methods such as restriction endonuclease analysis and DNA pairing are used for further classification into serotypes.

Resistance

Leptospires are very susceptible to heat, and are killed in 10 minutes at 50°C and in 10 seconds at 60°C. They are also sensitive to acid and are destroyed by gastric juice and bile. They are also readily destroyed by chlorine and most other antiseptics and disinfectants. They can survive for days in moist conditions at pH 6.8–8.

Pathogenicity

In natural reservoir hosts, leptospiral infection is asymptomatic but when transmitted to other animals, clinical disease may result. **It is a zoonotic disease.** Leptospires in water contaminated by the urine of carrier animals enter the body through cuts or abrasions on the skin or through the intact mucosa of the mouth, nose or conjunctiva. The incubation period is usually about 10 days (ranges from 2–26 days).

Clinical Manifestations

The clinical picture varies from mild to severe.

- It may be mild, with undifferentiated pyrexia.
- It could manifest as severe or fatal illness with hepatorenal damage (Weil's disease). In severe cases, the onset is acute, with rigor, vomiting, headache and conjunctival suffusion. The fever is irregular and usually subsides in about ten days. Jaundice occurs in about 10–20 per cent of cases by the second or third day. Purpuric hemorrhages sometimes occur on the skin and mucosa. Albuminuria is a constant feature (*Clinical case 2*).

Two clinical types, **icteric** and **non-icteric** are noted**.** A number of cases present as aseptic meningitis and, in some, abdominal symptoms predominate.

Leptospires are **seen in the blood during the acute phase of the disease** but can seldom be demonstrated after 8–10 days. They persist in the internal organs, and most abundantly in the kidneys, so they **may be demonstrated in urine in the later stages of the disease.**

Epidemiology

Leptospirosis is considered the most widespread of zoonoses on account of its presence in all continents except Antarctica. Pathogenic leptospires survive for long periods in the convoluted tubules of the kidneys in natural hosts, multiply and are shed in the urine. Animal carriers often excrete up to 100 million leptospires per mL of urine into contaminated water. In mud that is neutral or slightly alkaline, the leptospires survive for weeks. When

people come into contact with such water, the leptospires enter the body through abraded skin or mucosa and initiate infection.

Certain occupational groups such as **agricultural workers** in rice or cane fields, miners and sewer cleaners are exposed to the infection more often, and so leptospirosis is more common in them. Incidence rises following floods and waterlogging in urban areas.

> In India, leptospirosis occurs following the monsoons and floods. It is reported from South and West India. Andaman hemorrhagic fever due to leptospira is endemic in Andamans. The serovar *Portblairi* and serogroup *Sehgali* have been associated with leptospirosis in the island.

Laboratory Diagnosis

WHO's criteria to diagnose leptospirosis are based on three criteria known as the Faine's criteria. They include A—clinical data, B—epidemiological factors and C—laboratory findings. Faine's criteria are based on a scoring system with numerical scores being assigned to individual components. A score of part A or A + B = 26 or more and part A + B + C = 25 or more is diagnostic of leptospirosis.

Some of the clinical criteria include fever, headache, conjunctival suffusion, jaundice, etc. Epidemiological criteria include contact with contaminated water or animal and laboratory criteria positive for IgM by ELISA or MAT.

1. Microscopy

Examination of blood As leptospires disappear from blood after the **first week**, blood examination is helpful only in the early stages of the disease, before antibiotics are given.

Examination of urine Leptospires appear in urine in the **second week** of the disease and intermittently thereafter for 4–6 weeks. **The urine should be examined immediately after voiding as leptospires readily undergo lysis in acidic urine.**

2. Culture

Culture media Leptospires can be grown in media enriched with rabbit serum. Several liquid and semi-solid media, such as **Korthof's, Stuart's** and **Fletcher's** media, have been described. Semisynthetic media, such as **EMJH** (Ellinghausen, McCullough, Johnson, Harris), are now commonly

used. Leptospires are aerobic and microaerophilic. In semi-solid media, growth occurs a few millimetres below the surface in the form of a ring. Optimum temperature is 25–30°C and optimum pH 7.2–7.5.

Embroyonated eggs Leptospires may be grown on the chorioallantoic membrane of chick embryos. They may be demonstrated in the blood of allantoic vessels 4–5 days after inoculation.

Identification of isolates is a complicated procedure and is generally confirmed by one of the WHO/FAO Reference Laboratories.

3. **Animal inoculation** A simple method for obtaining cultures free of contaminants is to inoculate the material intraperitoneally in guinea pigs and culture the heart blood collected ten minutes later. Leptospires invade the bloodstream more rapidly than other bacteria. With virulent serotypes like *icterohemorrhagiae*, the animals develop fever and die within 8–12 days with jaundice and hemorrhage into the lungs and serous cavities.

4. **Serological diagnosis** Antibodies appear in serum towards the end of the first week of the disease and increase till the fourth week, declining thereafter.

- The broadly reactive or genus-specific tests identify leptospiral infection without indicating the exact infecting serovar. ELISA has been used to detect IgM and IgG antibodies separately, in order to indicate the stage of infection. A simple and rapid dipstick assay has been developed for the assay of leptospira-specific IgM antibody in human sera.
- The antigens for these tests are prepared from the non-pathogenic *L. biflexa* Patoc 1 strain. Some of the older tests include sensitised erythrocyte lysis (SEL), complement fixation, agglutination and indirect immunofluorescence. The type-specific tests identify the infecting serovar by demonstrating specific antibodies. Macroscopic and microscopic agglutination tests are used for this purpose.
    - **Macroscopic agglutination test** Here, formalinised suspensions of prevalent leptospira serovars are tested for macroscopic agglutination with serial dilutions of the test serum. **Cross-agglutinin absorption test** (**CAAT**) also

follows a similar principle. The serum is absorbed to make it more specific.

- **Microscopic agglutination test (MAT)** This uses live cultures of different serotypes, and agglutination is observed under the low-power, dark-field microscope. This test is more specific and is usually done only in reference laboratories. Due to the presence of some degree of cross-reaction between different serovars, agglutinin absorption tests may sometimes become necessary for accurate diagnosis.

These tests have been used in the surveillance of leptospirosis in endemic areas.

Rats are particularly important as they are ubiquitous and carry the most pathogenic serotype, *icterohemorrhagiae*. Field mice carry *grippotyphosa*, pigs *pomona* and dogs *canicola*.

Infection among animals is also transmitted by urinary contamination of water and fodder. Human beings are an aberrant or 'end' host. There is no evidence that human patients infect others. Infection in urban areas is perhaps due to overcrowding, unsanitary conditions, increasing rat population and the habit of walking barefoot.

Prophylaxis

General measures of prevention such as rodent control, disinfection of water and the wearing of protective clothing contribute to the prevention of leptospirosis. Vaccination has been attempted with some success in dogs, cattle and pigs. Immunity following vaccination or infection is serotype-specific. Vaccination has also been tried in persons at high risk such as agricultural workers.

Treatment

Leptospires are sensitive to penicillin and tetracycline but treatment should be started early in the course of the disease to be effective. Penicillin is given as IV, 1–2 million units six-hourly for seven days in serious cases. A mild Jarisch–Herxheimer reaction may occur in some. Doxycycline, 200

mg, orally given once a week, is effective in prophylaxis.

Key points

- Spirochetes are helical, slender, relatively long bacteria, which are widespread in nature. Spirochetes responsible for human diseases are:
    - *Treponema* species, which cause syphilis, yaws and pinta
    - *Borrelia* species, which cause Lyme disease and relapsing fever
    - *Leptospira* species, which cause leptospirosis
- Syphilis, which can present as primary, secondary and tertiary stages, can be acquired by venereal or congenital transmission.
- Primary and secondary syphilis can be diagnosed by a combination of microscopic and serological techniques. Dark-ground or phase-contrast microscopy reveal spirochetes with characteristic motility; alternatively, the specimen can be stained by silver impregnation and viewed under bright-field microscopy to demonstrate bacteria with distinctive morphology.
- Serological tests consist of non-specific (standard) tests such as VDRL (to detect anti-cardiolipin antibody) and specific (confirmatory) tests, such as fluorescent treponemal antibody (FTA) and *T. pallidum* hemagglutination (TPHA) tests to detect anti-*T. pallidum* antibody.
- *T. pertenue* causes yaws, *T. carateum* causes pinta and *T. pallidum* var endemicum causes endemic syphilis; these species found in tropical countries are transmitted by non-sexual means (mainly trauma).
- The genus *Borrelia* are gram-negative and strictly anaerobic. They are transmitted to humans following the bite of an insect vector (primarily lice or tricks).
- *Borrelia recurrentis* is responsible for the louse-borne or epidemic type of relapsing fever, with humans serving as the reservoir hosts.
- Lyme disease is a tick-borne illness and is caused by *Borrelia burgdorferi*; the disease occurs in the northern temperate zone, and rodents are the major reservoir (humans are possibly only accidental hosts).
- Laboratory diagnosis of relapsing fever includes dark-field microscopy of blood during febrile episodes to demonstrate the spirochetes, and detection of specific antibody to *B. recurrentis* by indirect immunofluorescence assay.
- The genus *Leptospira* comprises very thin, tightly coiled, obligately aerobic spirochetes with hooked ends, which possess a unique, flexuous type of motility. They can be cultivated in vitro. Serotypes of *Leptospira interrogans* cause leptospirosis, a zoonotic disease, while serotypes of *Leptospira biflexa* exist in water and soil as free-living organisms.
- *Leptospira* infections can occur as epidemic forms after floods and water stagnation in urban areas. Diagnosis is made by culture of blood, CSF and urine and serum for serological tests (microscopic agglutination test); a four-fold rise in convalescent titres is considered

positive. Other tests include dark-field examination of blood or urine; recently, rapid commercial tests (enzyme immunoassays) have been developed. Macroscopic slide agglutination test is a reference test for serological diagnosis.

Questions

Essays

1. Enumerate the sexually transmitted diseases and describe the laboratory diagnosis of syphilis.
2. Classify spirochetes pathogenic to humans and describe the laboratory diagnosis of any one of them.

Short Answers

1. Advantages and disadvantages of the VDRL test
2. Specific *T. pallidum* tests
3. Laboratory diagnosis of leptospirosis
4. Non-venereal treponematoses
5. Vincent's angina
6. Relapsing fever
7. Lyme disease
8. VDRL test
9. TPHA
10. Weil's disease

Part III: Bacteriology

Chapter 36

Chlamydia

Chapter navigation

- *Chlamydia trachomatis*
  - Inclusion conjunctivitis
  - Genital chlamydiasis
- Lymphogranuloma venereum
- *Chlamydophila pneumoniae*
- *Chlamydophila psittaci*

INTRODUCTION

Chlamydiae are obligate intracellular bacteria that infect humans, animals and birds. They exhibit tropism for squamous epithelial cells and macrophages of the respiratory and gastrointestinal tracts. They cause pneumonia, ocular infections and a broad range of sexually transmitted infections such as urethritis, cervicitis, lymphogranuloma venereum and others.

The chlamydial **cell wall** resembles that of gram-negative bacteria, consisting of an outer layer, outer membrane complex (which possibly confers rigidity and stability) and an inner layer. Lipopolysaccharide is present in the outer membrane complex, with a small amount of peptidoglycan. Unlike bacteria, however, **they do not have peptidoglycan cell walls**. They lack enzymes of the electron transport, survive in an intracellular environment and cannot be cultured on artificial media.

Classification

Taxonomically, chlamydiae have been divided into two distinct genera based on their molecular nature: (a) *Chlamydia* and (b) *Chlamydophila*.

Genus *Chlamydia* includes a single species, *Chlamydia trachomatis*.

Genus *Chlamydophila* includes two species:

- *Chlamydophila pneumoniae*
- *Chlamydophila psittaci*

A third species, *Chlamydophila pecorum*, has been created recently to include some strains affecting ruminants. However, from the medical perspective, they are still known as *Chlamydia*. They produce a wide range of diseases from respiratory to ocular to sexually transmitted diseases in humans.

Species differentiation is based on growth characters, nucleic acid profile, antigens, plasmids and the nature of the inclusion body.

- *C. trachomatis* strains form **compact inclusions with the glycogen matrix**. They are sensitive to sulphonamides and are natural parasites of humans, usually causing localised infections of the eyes and genitals.
- *C. psittaci* strains form **diffuse vacuolated inclusions without the glycogen matrix**, and are resistant to sulphonamides. They are natural parasites of birds and animals, capable of causing pneumonia and generalised infection in humans.
- *C. pneumoniae* is an exclusively human pathogen with no animal or avian host. It is a common cause of acute respiratory disease worldwide.

Morphology

Chlamydiae occur in two forms:

i) **Elementary body** is the extracellular, infective form. It is a spherical particle, 200–300 nm in diameter, with a rigid trilaminar cell wall similar to the cell walls of gram-negative bacteria, and an electron-dense nucleoid.

ii) **Reticulate body** (formerly called the 'initial body') is the intracellularly growing and replicative form, 500–1,000 nm in size. Its cell wall is fragile and pliable, leading to pleomorphism.

Growth Cycle

Infection is initiated by the attachment of the elementary body to the surface of a susceptible epithelial cell, followed by its endocytosis (Fig. 36.1). Inside the host cell, the elementary body lies within the endosome throughout its active growth cycle. By about eight hours, the **elementary body** within the endosome is converted to a **large reticulate body**. Binary fission starts in 12 hours, and by 20–24 hours, the progeny are converted to elementary bodies. The developing chlamydial microcolony within the host cell is called the **inclusion body**. The mature **inclusion body contains 100–500 elementary bodies,** which are ultimately released from the host cell.

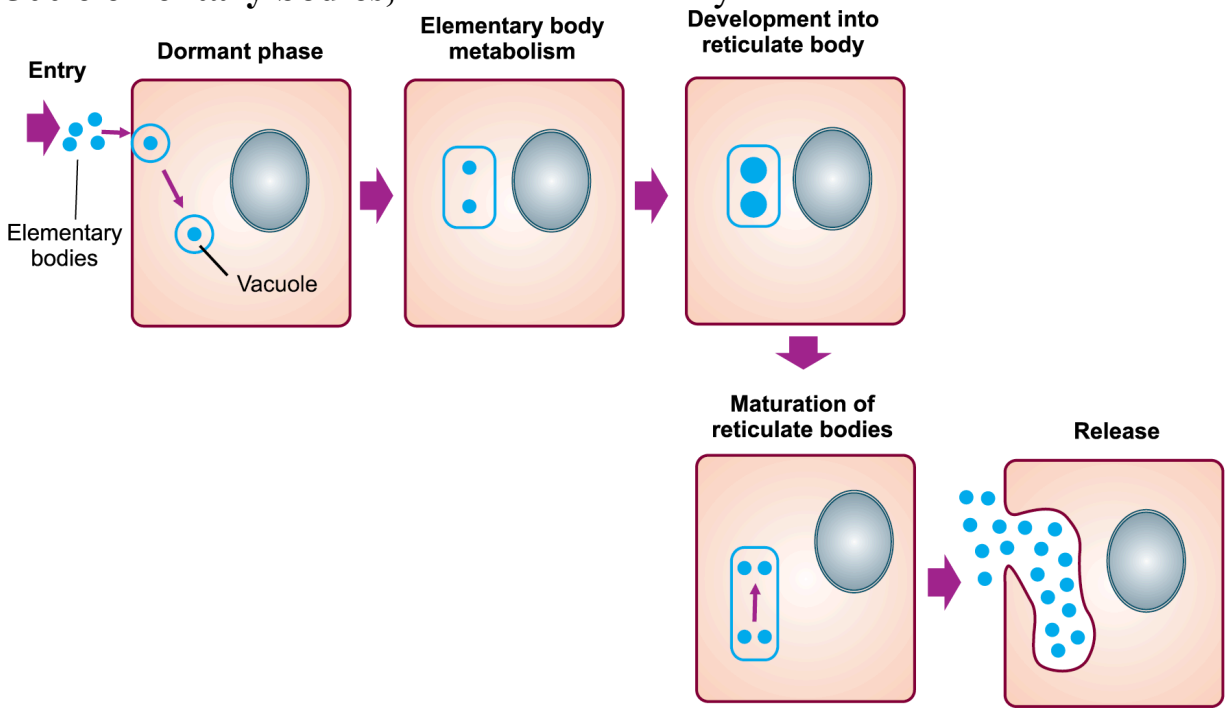


Fig. 36.1 Reproductive cycle of *Chlamydia*

During active intracellular growth, the *Chlamydia*-specific lipopolysaccharides accumulate on the host cell surface. This highly antigenic material induces inflammatory and immunological responses which contribute to the pathogenesis of chlamydial diseases.

Antigenic Properties

Chlamydiae possess three main kinds of antigens:

1. **Genus-specific antigen** This is a heat-stable antigen, common to all chlamydiae. It is a lipopolysaccharide similar to LPS of enteric gram-

negative bacilli and is present in all stages of the developmental cycle and can be identified by the complement fixation test.

2. **Species-specific protein antigen** The second type is present at the envelope surface. These antigens are present in all strains of chlamydial species. They help in classifying chlamydiae into species —*C. trachomatis, C. psittaci, C. pneumoniae* and *C. pecorum*.
3. **Serotype/var-specific antigen** The third kind helps in intraspecies typing, as it is found only in some members of a species. These antigens are located on the major outer membrane proteins (MOMP) and can be demonstrated by microimmunofluorescence (micro-IF) and are designated serotype or serovar.

Type-specific antigens are used to classify *Chlamydia* into three species, which are as follows:

i) *C. trachomatis* causes trachoma inclusion conjunctivitis (TRIC). The TRIC agent has been classified into:

- 12 serovars (a) A, B, Ba and C that cause blinding trachoma in endemic areas and (b) serovars D to K associated with the less serious ocular infection, inclusion conjunctivitis and various genital infections.
- 3 serovars L1, L2 and L3, which cause lymphogranuloma venereum (LGV) and hemorrhagic proctitis.

ii) *Chlamydia* (*Chlamydophila*) *pneumoniae* has one serotype. TWAR is a strain variant.

iii) *Chlamydia* (*Chlamydophila*) *psittaci*, the serological classification of which is complex. Many serotypes having been identified.

Human diseases associated with *Chlamydia* are listed in Table 36.1.

Table 36.1 Human diseases caused by chlamydiae

Species	Serotype*	Disease
C. trachomatis	A, B, Ba, C	Endemic blinding trachoma
C. trachomatis	D, E, F, G, H, I, J, K	Inclusion conjunctivitis (neonatal and adult) Genital chlamydiasis Infant pneumonia

C. trachomatis	L1, L2, L3	Lymphogranuloma venereum
C. pneumoniae	Only one serotype TWAR strain	Acute respiratory disease Outbreaks in young populations living in close quarters and in long-term care facilities

* Predominant types associated with the disease

Resistance

Chlamydiae are heat-labile, being inactivated within minutes at 56°C. They are susceptible to ethanol, ether and low concentrations of phenol and formalin. Infectivity is maintained for several days at 4ºC. They can be preserved frozen at –70ºC or lyophilised.

Laboratory Diagnosis

Four approaches are available for the laboratory diagnosis of chlamydial infections:

i) Microscopic demonstration of inclusion or elementary bodies
ii) Isolation of *Chlamydia*
iii) Demonstration of chlamydial antigen
iv) Demonstration of antibodies or hypersensitivity

1. **Clinical specimen** The type of specimen depends on the clinical type of infection:

- **Conjunctival material** is collected with a metal or special plastic impression spatula in ocular infections.
- **Urethral scrapings** are collected using a specially designed curette in genital infections; in women, cervical scrapings are also collected.
- **Urine sample** can also be collected. First, 20 mL of voided is collected to prevent dilution of the anterior urethral specimen.
- **Blood and sputum** are collected for microscopy and culture, and serum for serology in suspected psittacosis.
- **Material is aspirated from the bubo** (inguinal lymphadenitis) in suspected lymphogranuloma venereum.

2. **Microscopy**

- Chlamydiae are gram-negative but are stained better by the **Giemsa**, **Castaneda**, **Macchiavello** and **Gimenez** stains. Diagnosis of ocular infections, particularly in neonatal inclusion conjunctivitis, depends on the demonstration of inclusion bodies (Fig. 36.2).

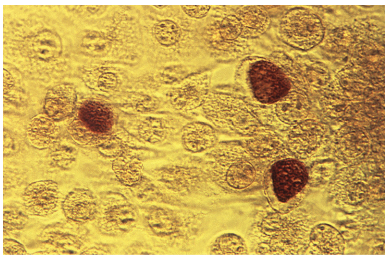


Fig. 36.2 Tissue culture showing chlamydial inclusion bodies (*Source:* Public Health Image Library, ID 3802 / Dr. E. Arum; Dr. N. Jacobs / CDC)

- **Lugol's iodine** Because of the glycogen matrix of *C. trachomatis* inclusions, they can be stained with iodine. Iodine-staining is not applicable in *C. psittaci* because its inclusions do not contain glycogen.
- **Immunofluorescence (IF)** A more sensitive and specific method of microscopic examination is immunofluorescence using monoclonal antibodies. IF can identify not only inclusions but also extracellular elementary bodies. IF is also useful in the examination of cervical or urethral specimens, which may contain elementary bodies but few intact intracellular inclusions.

3. **Isolation**

- **Tissue culture** Cell culture is the preferred mode of isolation. Many cell lines are susceptible but the McCoy and HeLa cells are the most commonly used. *C. trachomatis* strains vary in their infectivity to cell cultures. LGV strains grow well, while TRIC strains are less infective. Cell cultures used for isolation **are pre-treated by irradiation or chemicals such as 5-iodo-2-deoxyuridine or cycloheximide** to enhance chlamydial replication and facilitate the detection of inclusion bodies. Pre-treatment of cells with DEAE dextran or centrifugation after inoculation promotes contact between chlamydial particles and

the cell monolayer in shell vial cultures, thereby increasing the chances of isolation.

- **Embryonated chick egg inoculation** This can be done by inoculating the material into embryonated eggs. Chlamydia can grow in the yolk sac of 6–8-day-old chick embryos. However, this method is not widely applied now.
- **Experimental animals (mice)** Chlamydiae differ in their infectivity to mice. However, mice inoculation is no longer in use for isolation of chlamydia.

4. **Antigen detection** Demonstration of chlamydial antigens by immunofluorescence is commonly used as a rapid diagnostic test.

- The infected ocular or genital samples are smeared on a slide and stained with specific fluorescent conjugated antibody and examined under the fluorescence microscope. This method is rapid and as sensitive as culture.
- **ELISA** is the method preferred for screening as it enables rapid testing for the LPS antigen in a large number of specimens.
- **Molecular methods** like DNA probes, nucleic acid amplification techniques (NAAT) and ligase chain reaction have greatly increased the sensitivity and specificity of antigen detection. These tests are slowly replacing enzyme immune assay and cultures (which need viable organisms in the specimen).

5. **Antibody detection** Diagnosis by demonstration of antibody in serum may be done by the group-specific CF test or type-specific micro-IF. During chlamydial infection, specific antibodies are produced. First, the IgM antibodies (signifying primary infection), which persist for approximately two months and then IgG antibodies are formed. The **CF test** is used mainly in invasive chlamydial infections such as psittacosis and LGV. A four-fold rise in titres is diagnostic. **A single CF antibody test is not diagnostic of psittacosis or LGV unless the titre is high—1:64** or greater.

- **Micro-IF** can test IgG and IgM antibodies separately. Titres of 1:8 or greater are usual in infected persons. The initial antibody

response is IgM, which is replaced by IgG after about a month. **Recurrent infection with the same serotype induces only IgG response**. Four-fold rise in IgG titre or the presence of IgM antibody is suggestive of past or recent infections.

High-titre antibodies are usually seen only in infant pneumonia, salpingitis and LGV.

CHLAMYDIA TRACHOMATIS

C. trachomatis is a leading cause of ocular and genital infections worldwide.

Trachoma

Trachoma is a chronic keratoconjunctivitis, characterised by follicular hypertrophy, papillary hyperplasia, pannus formation and in the late stages, cicatrisation
(Fig. 36.3). The name trachoma is derived from the Greek word *trakhus* (rough), referring to the roughness of the conjunctiva in the disease. Halberstaedter and Prowazek transmitted the infection to orangutans to demonstrate in vitro transmission in the experimental animal.

Fig. 36.3 Inflamed conjunctival membrane lining the inside of both the upper and lower lids due to inclusion conjunctivitis, caused by *Chlamydia trachomatis* (*Source:* Public Health Image Library, ID 15193 / Susan Lindsley / CDC)

Pathogenicity

Infection is transmitted to the eyes by fingers or fomites. Flies may transmit the infection mechanically.

Trachoma is suspected when any of the following are present

- Five or more follicles >0.5 mm in diameter on the tarsal

conjunctivae
- Conjunctival scarring
- Limbal follicles or Herbert pits
- Corneal neovascularisation and granulation tissue formation

Stages

The World Health Organization has classified trachoma into the following 5 stages:

1. Trachomatous inflammation follicular
2. Trachomatous inflammation intense
3. Trachomatous scarring of the tarsal conjunctiva
4. Trachomatous trichiasis
5. Trachomatous corneal opacity

Laboratory Diagnosis

1. **Staining** The characteristic inclusions (**Halberstaedter–Prowazek** or **HP bodies**) may be demonstrated in conjunctival scrapings after staining them by Giemsa, Castaneda or Macchiavello methods (Fig. 36.4). Because they possess a glycogen matrix, they take up iodine, which enhances the sensitivity.

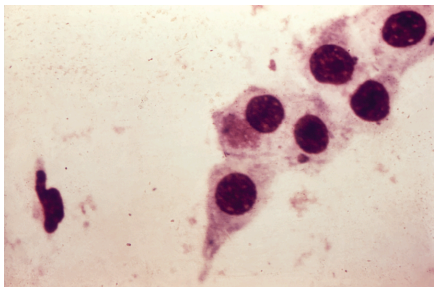


Fig. 36.4 *Chlamydia trachomatis* intracytoplasmic inclusion bodies taken from a urethral scraping (*Source:* Public Health Image Library, ID 2295 / Dr. Wiesner, Dr. Kaufman /CDC)

2. **Culture**

Tissue culture Chlamydia are isolated using the following stationary-phase cells (non-replicating cells):

- McCoy cells rendered non-replicating by irradiation or antimetabolites
- HeLa cells treated with DEAE dextran

The inoculum has to be made adherent on to the cells by centrifugation up

to 15,000 g in shell vials to obtain good growth. The presence of growth is identified by immunofluorescence.

Chlamydia may also be grown in the **yolk sac** of 6–8-day-old eggs.

Treatment

Local application and oral administration of erythromycin and tetracycline or other suitable antibiotics should be continued for several weeks. A single-dose azithromycin treatment has been used with good results.

Epidemiology and Control

Trachoma is worldwide in distribution and about 500 million people are estimated to be affected. It is particularly prevalent in the developing nations because of overcrowding and unhygienic conditions. It is endemic in the Middle East, Africa, India and the Far East. Application of '*surma*' or '*kajal*' from a common source has been implicated in rural Indian communities. Control of the disease involves mass education and chemotherapy.

Inclusion Conjunctivitis

Clinical Case 1

The mother of a five-day-old neonate complained of a thick, yellowish discharge in both the eyes of her child. The child was afebrile and able to feed properly. Gram-staining of the exudate did not reveal any bacteria. A Giemsa-stained smear of the exudates was positive for a large cytoplasmic inclusion. A diagnosis of inclusion conjunctivitis was made and the child responded to a course of erythromycin.

Inclusion blennorrhea, the neonatal form of inclusion conjunctivitis, develops when the infant is infected in the birth passage. It usually becomes apparent between
5 and 12 days after birth. It has a high incidence of micropannus, conjunctival scars and late recurrences. These can be prevented by the local application of antibiotics (*Clinical case 1*).

In the adult form of the disease, **swimming pool conjunctivitis**, there is follicular hypertrophy with scanty, non-purulent discharge. Infection is presumably due to contamination with chlamydia from the genital secretions of bathers or the patient's own genital secretion may more often

be the cause of infection of the eye (due to *Chlamydia* serotypes D–K).

Infant Pneumonia

C. trachomatis can cause pneumonia in infants, usually around 4–16 weeks of age. Characteristically, they develop prominent respiratory symptoms with cough and wheezing but fever and toxicity are minimal. Conjunctivitis often precedes pneumonia. A high IgM titre to the infecting serovar is diagnostic. Immune response is believed to play a role in the pathogenesis of this condition.

Genital Infections

Clinical Case 2

A 20-year-old male presented to an STD clinic with complaints of urethral discharge and dysuria for two weeks. The patient was subjected to laboratory investigations and a Gram smear of the discharge showed plenty of pus cells but no bacteria; Giemsa stain showed epithelial cells with intracytoplasmic inclusion bodies. Culture was negative. The chlamydial antigen detection test was positive.
A diagnosis of non-gonococcal urethritis (NGU) was made and treatment with tetracycline was given for seven days.

C. trachomatis causes two types of genital infections:

i) Urogenital syndromes caused by the oculogenital serotypes D to K, collectively referred to as genital chlamydiasis and
ii) LGV caused by serotypes L1, L2 and L3.

Genital chlamydiasis

Chlamydial infections have become the most common sexually transmitted disease worldwide. Their clinical spectrum parallels that of gonococcal infections. The two may often co-exist (*Clinical case 2*).

- In men, urethritis (**non-gonococcal urethritis** or **NGU**), epididymitis, proctitis, conjunctivitis and **Reiter's syndrome** may be caused.

Reiter's syndrome is a triad of recurrent conjunctivitis, polyarthritis and urethritis or cervicitis, associated with many infections but most commonly, with *C. trachomatis*.

- Women develop acute urethral syndrome, bartholinitis, mucopurulent cervicitis, endometritis, salpingitis, pelvic inflammatory disease, conjunctivitis, perihepatitis (**Fitz-Hugh–Curtis syndrome**) and Reiter's syndrome. Genital chlamydiasis may cause premature deliveries, perinatal morbidity and postpartum fever. Untreated infection may lead to pelvic inflammatory disease (PID), infertility and potentially fatal tubal pregnancy.

In India, chlamydial infection has been reported in 20–30 per cent of women with mucopurulent cervicitis and 30–60 per cent of those with salpingitis and pelvic inflammatory disease.

In the laboratory, diagnostic findings are the presence of neutrophils (more than 4 per oil immersion field in urethritis and more than 30 in cervicitis) in the absence of gonococcal infection. Confirmation is by antigen detection by micro-IF, ELISA and by molecular techniques.

Lymphogranuloma venereum

This sexually transmitted disease is characterised by suppurative inguinal adenitis.

Also known as **lymphogranuloma inguinale**, it is caused by the **LGV serovars L1, L2 and L3** of *C. trachomatis* with predilection for regional lymph nodes. The main pathogenic features are as follows:

1. The **primary lesion** is a small, painless, papulovesicular lesion appearing on the external genitalia (or rarely, extragenital sites) after an incubation period of three days to five weeks.
2. The **secondary stage**, two weeks later, spreads from lymphatic to the draining lymph nodes.

- Inguinal lymph nodes are involved in men.
- In women, the intrapelvic and pararectal nodes are involved.
- Women and homosexual men may develop hemorrhagic proctitis with regional lymphadenitis.
- The nodes enlarge, suppurate, become adherent to the skin and break down to form sinuses discharging pus.

- Metastatic complications may sometimes occur, with the involvement of the joints, eyes and meninges.

3. The **tertiary stage** is chronic, lasting for several years, with the sequelae of scarring and lymphatic blockage. Late sequelae are more distressing in women as they lead to rectal strictures and disfigurement of the vulva (esthiomene).

The primary lesion usually goes unnoticed and the disease is usually first seen at the stage of inguinal adenitis (bubo). Smears of material aspirated from the buboes may show the elementary bodies (**Miyagawa's granulo-corpuscles**). The sensitivity of microscopic diagnosis is very low. Isolation of chlamydia is done by cell cultures. LGV patients develop high titres of circulating antibodies, with titres of 1:512 or more in micro-IF test which is diagnostic.

Treatment is with doxycycline, 100 mg, twice a day for at least three weeks. Erythromycin or azithromycin can also be used.

CHLAMYDOPHILA PNEUMONIAE

C. pneumoniae has been established as a human pathogen that causes respiratory illness. Only one serovar has been demonstrated. TWAR (Taiwan Acute Respiratory strain) is a variant. This appears to be an exclusively human *Chlamydia,* transmitted from human to human without any avian or animal host. *C. pneumoniae* is a common cause of respiratory disease in older children and adults worldwide. Its clinical spectrum includes pharyngitis, sinusitis, bronchitis and community-acquired pneumonia. Clinical findings are as atypical pneumonia, similar to the clinical picture of *Mycoplasma* pneumonia. Sometimes, severe lower respiratory tract infection may occur. Considerable interest has been aroused by recent reports linking *C. pneumoniae* with **atherosclerosis** and **coronary, carotid** and **cerebral arterial diseases**.

Laboratory diagnosis is mainly by microimmunofluorescence test, which is the most sensitive method. It demonstrates IgM (after 1–2 weeks) or IgG (6–8 weeks later). Microscopy to detect elementary bodies is not sensitive. Culture on HeLa 229 or MacCoy cell lines incorporated with cycloheximide can be used to isolate

C. pneumoniae. This is done by shell vial culture with centrifugation of the inoculum onto the cell lines. Nucleic acid amplification methods have also been tried.

Treatment is by macrolides (azithromycin, clarithromycin) and tetracyclines for one week.

CHLAMYDOPHILA PSITTACI

Psittacosis

Psittacosis is a disease of parrots (*psittacos* means parrot) and other psittacine birds, transmissible to human beings. It is caused by ***Chlamydophila psittaci***.

Human infections are mostly occupational, as seen in poultry workers, pigeon farmers, pet shop owners, bird fanciers and veterinarians. Infection is by inhalation. Rare cases of infection by parrot bite have been reported. The high infectivity of psittacosis is indicated by the frequency of laboratory infections. Strains from parrots and turkeys are more virulent than those from other avian sources.

Pathogenicity

The incubation period is about 10 days. Clinical disease varies from a mild influenza-like syndrome to fatal pneumonia. The common complications resulting from this infection are the following:

- Pneumonia (the usual clinical manifestation)
- Septicemia
- Meningoencephalitis
- Endocarditis, pericarditis and arthritis
- Typhoid-like syndrome

Laboratory Diagnosis

Chlamydiae can be isolated from blood during the early stages of the disease and from sputum later on. Infected cells, including alveolar macrophages from patients, show inclusion bodies (**Levinthal–Cole–Lillie** or **LCL bodies**). These differ from *C. trachomatis* inclusions in being more

diffuse and irregular, **not stained by iodine and not inhibited by sulphadiazine or cycloserine**. Serological diagnosis may be made by the group-specific complement fixation test or type-specific micro-immunofluorescence test.

Key points

- Chlamydiae, which lack cytochromes, are obligate intracellular bacteria.
- The elementary body is the metabolically inert, extracellular infectious form of *Chlamydia* and the reticulate body is the metabolically active, dividing, intracellular form.
- Three species of *Chlamydia* are known to cause human infections—*C. trachomatis, C. pneumoniae* and *C. psittaci*.
- There are three biovars of *C. trachomatis*, of which the *trachoma* biovar and the *lymphogranuloma venereum* (LGV) biovar preferentially infect humans.
- Chlamydiae possess antigens that are present in all stages of the developmental cycle and are used in the indirect fluorescent antibody serological test:
    - Group-specific polysaccharide antigen (common to both *C. psittaci* and *C. trachomatis*) is useful for the diagnosis of psittacosis and LGV.
    - Species-specific proteinaceous antigens are used to differentiate *C. psittaci* from *C. trachomatis.*
    - Type-specific antigens are used to classify *C. trachomatis* into two broad biovars (biological variants) which cause (i) trachoma inclusion conjunctivitis (TRIC) and (ii) LGV.
- Cell-mediated immunity is very important for protection against *Chlamydia*.
- Clinical features of chlamydial infection include:
    - Ocular infection with *C. trachomatis.*
    - Sexually transmitted disease, manifesting:
        - in women as urethritis or cervicitis; complications include acute or chronic salpingitis, pelvic inflammatory disease and perihepatitis.
        - in men, as urethritis; complications include acute unilateral epididymitis, with sterility as the end result.
        - in the neonates, as conjunctivitis and, later, interstitial pneumonia.
- Reiter's syndrome is associated with male and female NGU.
- *C. pneumoniae* causes pneumonia.
- Diagnosis of chlamydial infections can be done by:
    - Microimmunofluorescence to demonstrate IgM and IgG antibodies
    - Isolation in irradiated or cycloheximide-treated HeLa 229 and McCoy cells lines or yolk sacs of embryonated hens' eggs
    - Serological tests based on group-specific, species-specific and type-specific chlamydial antigens in indirect fluorescent antibody technique or ELISA

- Treatment of patients and their partners with antibiotics like macrolides and tetracycline is important.

Questions

Essay

1. Classify chlamydial infections that occur in humans. Describe the pathogenesis of inclusion conjunctivitis.

Short Answers

1. Reticulate body of *Chlamydia*
2. Elementary body of *Chlamydia*
3. Lymphogranuloma venereum
4. TRIC agents
5. Psittacosis

Part III: Bacteriology

Chapter 37

Mycoplasma and Ureaplasma

Chapter navigation

- *Mycoplasma*
- *Ureaplasma urealyticum*
- Gential infections
- *Mycoplasma* as cell culture contaminants

INTRODUCTION

Mycoplasma and *Ureaplasma* are the smallest free-living bacteria that can be grown in a cell-free medium. They cause two categories of infections in humans—pneumonia caused by *M. pneumoniae* and urogenital infections by *Ureaplasma urealyticum, M. hominis* and *M. genitalium.* Mycoplasmas may be saprophytic, parasitic or pathogenic. More than 100 species of mycoplasma are known to cause disease in a variety of mammalian, insect and plant hosts. About 16 species belonging to three families are associated with human infection or colonisation (Table 37.1).

MYCOPLASMA

Mycoplasmas are bacteria that are devoid of cell walls and hence are highly pleomorphic, with no fixed shape or size. They lack cell wall precursors like muramic acid and diaminopimelic acid. The cells are bound by a soft trilaminar membrane containing sterols. They have a specific requirement of sterols for growth and membrane synthesis.

Mycoplasmas are chemo-organotrophs, their metabolism being mainly fermentative.

They were referred to as '**pleuropneumonia-like organisms**' **(PPLO)**, a term that has now been replaced with *Mycoplasma* (*myco*, from the fungus-like form of the branching filaments; *plasma*, denoting their plasticity of

shape). These organisms can pass through filters.

Morphology

Mycoplasma occur as granules and filaments of various sizes. They may be coccoid, balloon-, disc-, ring- or star-shaped forms; the filaments are slender and of varying length and show branching. Multiplication is by binary fission. A distinctive feature seen in some species is a bulbous enlargement with a differentiated structure of the tip, by means of which the organisms attach to suitable host cells carrying neuraminic acid receptors. They may be responsible for the hemadsorption shown by some species.

Mycoplasmas are gram-negative but are better stained by Giemsa stain.

Classification

Mycoplasma contains two genera of human importance (Table 37.1).

1. Genus *Mycoplasma*, which utilises glucose or arginine but does not split urea.
2. Genus *Ureaplasma*, which hydrolyses urea.

Table 37.1 Mycoplasmas responsible for human infections or colonisation: diseases, species and site of colonisation

Established pathogen	Infections	Non-pathogenic	Site of colonisation
M. pneumoniae	Pneumonia	*M. orale, M. buccale, M. salivarium, M. faucium*	Oropharynx
M. hominis and *U. urealyticum*	Genital infections	*M. fermentans, M. genitalium, M. penetrans*	Genital tract

Antigenic Properties

Mycoplasmal surface antigens are mainly **glycolipids** and **proteins**. Serological tests such as complement fixation, agglutination, passive hemagglutination, ELISA and immunofluorescence have been used to detect antibodies in sera:

- Glycolipid antigens are identified by complement fixation.
- Protein antigens are detected by ELISA.

- The **growth inhibition test** is based on the ability of antisera to specifically inhibit the growth of the homologous species on solid media.

Resistance

Mycoplasmas can be destroyed at 45°C in 15 minutes. They are can be lysed by surface-active agents and lipolytic agents such as taurocholate and digitonin. They are **resistant to penicillin and cephalosporin** as well as to lysozymes that act on bacterial cell walls but are sensitive to tetracycline, erythromycin and other macrolides as well as many other antibiotics.

Pathogenicity

Pathogenic mycoplasma exhibit host specificity. They generally produce infections by adhering to the mucosa of the respiratory, gastrointestinal and genitourinary tracts.

Mycoplasma cause two types of disease in humans: pneumonia and genital infections.

1. **Mycoplasmal pneumonia Primary atypical pneumonia (PAP**) is caused by *M. pneumoniae*. The disease is typically tracheobronchitis. Acute pharyngitis is uncommon and only a third of patients develop pneumonia. The incubation period is 1–3 weeks.

Onset is gradual, with fever, malaise, headache and sore throat. Paroxysmal cough may occur with **blood-tinged sputum.** The disease is characterised by minimum respiratory signs on physical examination but marked radiological evidence of consolidation, which is usually patchy, involving one of the lower lobes, starting at the hilum and fanning out to the periphery (Fig. 37.1). The disease is usually self-limiting, with recovery in 1–2 weeks. **Bullous myringitis** and **otitis** are common complications. Meningitis, encephalitis and hemolytic anemia are other complications.

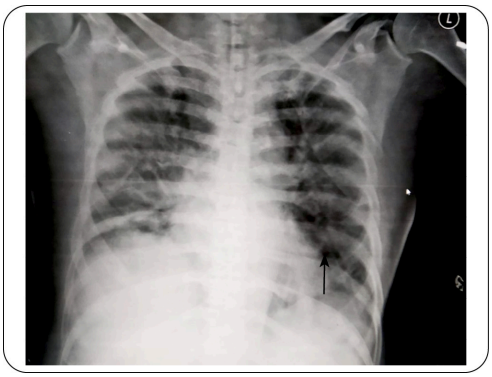


Fig. 37.1 Primary atypical pneumonia due to *M. pneumoniae* showing consolidation, involving one of the lower lobes (*Source:* Dr. Aneesh Basheer, Department of Medicine, Pondicherry Institute of Medical Sciences, Puducherry)

The disease is found worldwide and at all ages. Transmission is by droplets of nasopharyngeal secretions. The mycoplasma may remain in the throat for two or more months after recovery from the disease.

2. **Genital infections** are caused by *Mycoplasma hominis*, which has a similar spectrum as *Ureaplasma urealyticum*.

Laboratory Diagnosis

1. **Specimens** For isolation, throat swabs or respiratory secretions are inoculated into transport media (Fig. 37.2) to prevent drying and bacterial overgrowth.

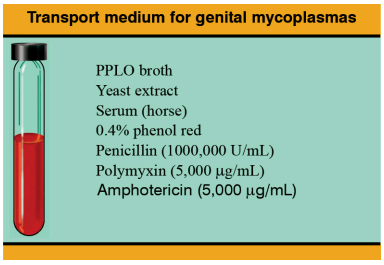


Fig. 37.2 PPLO medium for transport of genital specimen and growth of *Mycoplasma* (*Source:* Public Health Image Library, ID 15466 / Dr. Francis Forrester / CDC)

2. **Culture** Mycoplasma may be cultivated in **liquid** or **semi-solid media**. They are generally facultative anaerobes, growth being better aerobically. They grow within a temperature range of 22–41°C, optimally at 35–37°C. Media for cultivating mycoplasma are enriched with 20% horse or human serum and yeast extract. Penicillin and thallium acetate are added as selective agents. The high concentration of serum is necessary as a source of cholesterol and other lipids. Some of the notable media are **PPLO**

(pleuropneumonia-like organism) medium and **Hayflick medium**, which have similar constituents as mentioned above.

The colonies are seen directly on the agar by cutting a block of the medium and placing it on a slide with a coverslip coated with dried alcoholic solution of methylene blue and azure. Colonies appear very small at first (10–100 ∝m), growing slowly after incubation for 2–6 days to about 10–600 ∝m. The colony has a '**fried egg**' **appearance**, consisting of a central, opaque and granular area of growth extending into the depth of the medium, surrounded by a flat, translucent peripheral zone (Fig. 37.3). Colonies may be seen with a hand lens but are best studied after staining by the **Dienes method**. The colonies may be embedded in the agar.

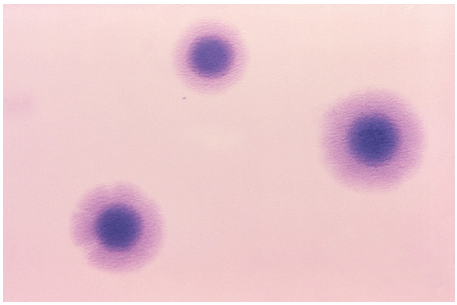


Fig. 37.3 'Fried egg' appearance of *Mycoplasma hominis* **and T-strain** *Mycoplasma* **isolates, which have been grown on agar medium (***Source:* **Public Health Image Library, ID 11024 / Dr. E. Arum; Dr. N. Jacobs / CDC)**

M. pneumoniae produces hemolysis and agglutinates guinea pig erythrocytes. Colonies also exhibit hemadsorption (they adsorb erythrocytes), which is enzymatic and occurs at 37°C. Subsequently, the cell receptors are destroyed by neuraminidase. Growth can also be screened by **tetrazolium reduction test** (mycoplasma colonies reduce the colourless tetrazolium to red-coloured formazan).

In a **liquid medium**, no turbidity is appreciated while pleomorphic forms are found in smears from the medium.

3. **Molecular methods** As isolation is difficult and delayed, PCR assay including multiplex PCR are used; they are rapid and specific.

4. **Serology** Serological diagnosis may be made by specific and non-specific tests.

- **Specific tests** using mycoplasmal antigens, which include immunofluorescence, hemagglutination inhibition and growth inhibition are the most sensitive tests. Complement fixation and indirect hemagglutination tests are less sensitive.
- **Non-specific tests**
    - **Streptococcus MG test** This is carried out by mixing serial dilutions of the patient's unheated serum and a heat-killed suspension of *Streptococcus* MG-intermedius (a strain of *S. milleri* anginosus group) and observing agglutination after overnight incubation at 37°C. A titre of 1:20 or over is considered suggestive.
    - **Cold agglutination test** is based on the presence of macroglobulin antibodies in PAP, which agglutinate human group O cells at a low temperature.
    - Serial dilutions of the patient's serum are mixed with an equal volume of 0.2% washed human O group erythrocytes. Clumping is observed after keeping it at 4°C overnight. The clumping dissociates at 37°C. A titre of 1:32 or over is suggestive. Demonstration of rise in titre in paired serum samples is more reliable. The indirect Coombs test may also be positive in some cases.

The patient's blood sample should not be refrigerated prior to separation of the serum as the agglutinins are readily absorbed by the homologous erythrocytes at low temperatures.

Mycoplasmas tend to cause severe and prolonged infections in HIV-infected and other immunodeficient subjects.

Treatment

Severe infections can be treated with doxycycline or tetracycline. Erythromycin, clarithromycin or azithromycin can also be used. As mycoplasma infections usually resolve on their own, antibiotic treatment of mild symptoms is not always necessary.

UREAPLASMA UREALYTICUM

Some strains of *Mycoplasma* frequently isolated from the urogenital tract of human beings and animals form very tiny colonies, generally 15–50 ∝m in size. They are called **T strain** or T form mycoplasmas which have been reclassified as *Ureaplasma urealyticum*.

- They hydrolyse urea, which is an essential growth factor in addition to cholesterol. Human T strain mycoplasmas have been associated with genital tract infection.
- They are the second most common cause of **non-gonococcal urethritis (NGU)** following *Chlamydia trachomatis.*

Genital Infections

They are caused by *M. hominis* and *U. urealyticum* and are transmitted by sexual contact. Specific infections are:

- **In women**—acute salpingitis, pelvic inflammatory disease, cervicitis and vaginitis. They are also associated with infertility, abortion, postpartum fever, chorioamnionitis and low birth weight of infants.
- **In men**—urethritis, proctitis, balanoposthitis and Reiter's syndrome, i.e., a combination of arthritis (usually involving the knee joints), conjunctivitis and urethritis occurring after a urogenital infection, in this case, mycoplasma infection.

Treatment

Azithromycin and moxifloxacin are advocated to treat NGU; they perform better than tetracycline and doxycycline which show some clinical response. *Ureaplasma*, however, can be resistant even to these agents.

Mycoplasmas and L-forms of bacteria Cell wall-deficient forms (L-forms, protoplasts and spheroplasts) are often confused with mycoplasma. L-forms do not initiate disease but may be important in bacterial persistence during antibiotic therapy and subsequent recurrence of infection.

Mycoplasma as Cell Culture Contaminants

Continuous cell cultures maintained in laboratories have been found to be

contaminated with different species of mycoplasma. The contamination may originate from the laboratory technologist or from animal sera or trypsin used in cell culture. Contamination generally does not produce cytopathic effects but may interfere with the growth of viruses in these cell cultures and misleading results in serological tests. They are difficult to eradicate.

All material used for cell culture have to be autoclaved at least for 20 min at 20 lbs pressure to kill the organism.

Key points

- Mycoplasmas are the smallest known free-living organisms. They have no cell wall but are bound by a typical three-layered cell membrane. They have pleomorphic morphology and may occur as spheres (0.3–0.5 µm in size) or as filaments. The cells are gram-negative but are better stained with Giemsa stain. They grow on cell-free media and require sterols for growth.
- Mycoplasmal (primary atypical) pneumonia is caused by *Mycoplasma pneumoniae*.
- *M. hominis* and *Ureaplasma urealyticum* may cause non-gonococcal urethritis as well as pelvic inflammatory disease in women.
- The growth on complex media resembles a 'fried egg'.
- In mycoplasmal pneumonia, the diagnosis is usually made by non-specific tests like the demonstration of cold agglutinins to human red blood cells. Growth inhibition tests are specific.
- Treatment with erythromycin and tetracycline is usually effective.
- *Ureaplasma urealyticum* causes NGU.

Questions

Short Answers

1. Primary atypical pneumonia
2. Laboratory diagnosis of *Mycoplasma pneumoniae* infections
3. Genital infections caused by *Mycoplasma*

Part III: Bacteriology

CHAPTER 38

Rickettsiae and Related Bacteria (Arthropod-Borne Bacterial Infections)

Chapter navigation

- Rickettsiaceae—
  - Genera: *Rickettsia* and *Orientia*
- Anaplasmatacea—
  - Genera: *Ehrlichia*, *Wolbachia*, *Anaplasma* and *Neorickettsia*
- Other allied arthropod-borne bacterial agents
  - Genera: *Bartonella* (*Rochalimaea*), *Grahamella*
  - Genera: *Coxiella*, *Rickettsiella*, *Piscirickettsia*
  - Genera: *Eperythrozoon*

RICKETTSIAE

The family Rickettsiaceae includes a diverse group of organisms that share the common features of intracellular growth and transmission by bloodsucking arthropod vectors (lice, fleas, ticks and mites). It is named after Howard Taylor Ricketts who discovered spotted fever rickettsia (1906) and died of typhus fever, which he contracted during his studies.

Characteristics

Rickettsiae are small, gram-negative bacilli. They cannot be seen by the ordinary light microscope and are obligate intracellular parasites, two characteristics in which they show similarity to viruses. However, they have many features of bacteria:

- Cell wall made of peptidoglycan
- Metabolic enzymes
- Both DNA and RNA
- Reproduction by binary fission
- Susceptibility to antibacterial agents

Classification

- Rickettsiaceae—Genera: ***Rickettsia*** **and** ***Orientia*** (comprise several human pathogens)
- **Anaplasmatacea—**Genera: ***Ehrlichia*****,** *Wolbachia*, *Anaplasma* and *Neorickettsia*

Of the genera, few are pathogenic to humans and have been highlighted.

Other allied arthropod-borne bacterial agents which have similar epidemiological and clinical features are dealt with in this chapter and include the following:

- Genera: ***Bartonella*** (*Rochalimaea*), *Grahamella*
- Genera: ***Coxiella***, *Rickettsiella*, *Piscirickettsia*
- Genera: *Haemorickettsiela* and *Eperythrozoon*

GENUS RICKETTSIA

Morphology

In smears from infected tissues, rickettsiae appear as pleomorphic coccobacilli, 0.3–0.6 × 0.8–2 ∝m in size. Structurally, they resemble gram-negative bacteria but do not take up the Gram stain well. They stain bluish-purple with the Giemsa and Castaneda stains and deep red with the Machiavello (Fig. 38.1) and Gimenez stains.

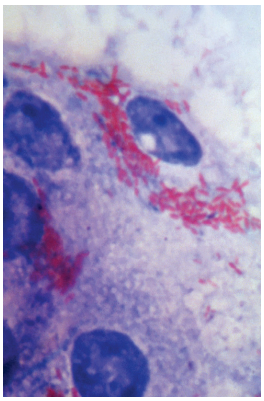


Fig. 38.1 *Rickettsia prowazekii* in a yolk sac smear specimen, processed using the Machiavello staining technique (*Source:* Public Health Image Library, ID 19028 / Cornelius B. Philip, National Institute of Allergy and Infectious Diseases, Rocky Mountain Lab., Hamilton, Montana / CDC)

Under the electron microscope, rickettsiae are seen to have a three-layered cell wall, a trilaminar plasma membrane and an outer slime layer. They are **obligate intracellular bacteria,** hence cannot be grown in artificial media. They are grown in tissue culture, embryonated eggs and in animals. Rickettsiae do not grow in cell-free media. So, growth in vitro is not possible. They grow in the cytoplasm of infected cells. In spotted fever, rickettsiae grow in the nucleus as well. They grow best in cells that are not metabolising actively. The optimum temperature for growth is 32–35°C.

They are readily cultivated in the yolk sac of developing chick embryos, as first shown by Cox. They also grow on mouse fibroblast, HeLa, HEp-2, Detroit 6 and other continuous cell lines

Antigenic Structure

Rickettsiae have species- and group-specific antigens. The immunodominant antigens are:

- **Surface protein antigens** (SPA) of *R. prowazekii* and *R. typhi* (typhus group) have both species-specific and cross-reactive epitopes.
- **Outer membrane proteins** (OMP) A and B are dominant in spotted fever rickettsiae. The former is a species-specific antigen that acts as an adhesin for host cells, and the latter shows limited cross-reaction with SPA of typhus rickettsiae.

- **Alkali-stable polysaccharide** found in some rickettsiae and in some strains of the *Proteus* bacilli is the third surface antigen.

Sharing of antigens between rickettsiae and proteus is the basis for the **Weil–Felix reaction** used for the diagnosis of rickettsial infections by the demonstration of agglutinins to *Proteus* strains OX 19, OX 2 and OX K.

Resistance

Rickettsiae are rapidly destroyed at 56°C and at room temperature. They are **preserved in skimmed milk** or a suspending medium containing sucrose, potassium phosphate and glutamate (**SPG medium**).

Pathogenesis

Reservoir host ↔ Vector ↔ Human being (often an accidental host)

Rickettsiae are transmitted to humans through the bite or feces of arthropod vectors from reservoir hosts. In humans, rickettsiae multiply locally and enter the bloodstream. They become localised chiefly in the vascular endothelial cells, which degenerate and cause thrombus formation, leading to partial or complete occlusion of the vascular lumen. The overall pathological features of the rickettsial diseases are similar in that they cause **acute febrile illness**, characterised by septicemia with **maculopapular rash** and fever; there may occasionally be **hemorrhage** and fatalities may occur in about 20 per cent of untreated cases. The distribution of the affected sites is shown in Fig. 38.2.

The long survival of rickettsiae in organs and lymphatic tissues of infected humans and animals is of importance in the epidemiology of some rickettsial diseases. *Rickettsia* is the causative agent of two groups of diseases:

1. Typhus fevers caused by *R. prowazekii*
2. Spotted fevers caused by *R. rickettsii*

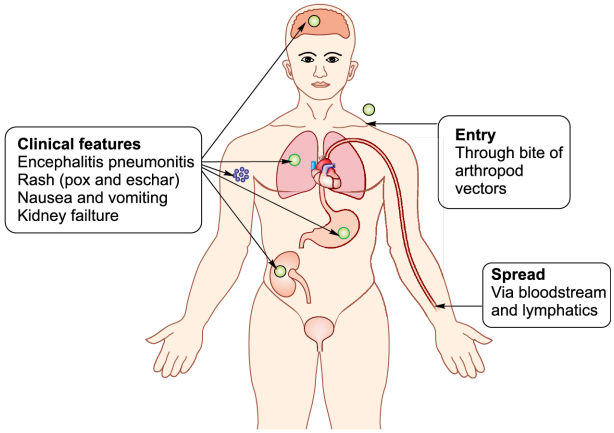


Fig. 38.2 Organ-specific clinical features and distribution of some rickettsial diseases in humans

Typhus Fever Group

This group of diseases includes the following (Table 38.1):

1. Epidemic typhus
2. Recrudescent typhus (Brill–Zinsser disease)
3. Endemic typhus

Table 38.1 Human diseases caused by the *Rickettsia* and *Orientia* species

Group	Species	Disease	Vector	Vertebrate reservoir	Distribution
Typhus	*R. prowazekii*	Epidemic typhus	Louse	Human beings	Worldwide
		Brill–Zinsser disease	Louse	Human beings	America, Europe, Australia
Spotted fever group	*R. typhi*	Endemic typhus	Rat flea	Rat	Worldwide
	R. felis	Endemic typhus	Cat flea	Opossum	USA
	R. rickettsii	Rocky Mountain spotted fever	Tick	Rabbit, dog, small rodents	North America
	R. siberica	Siberian tick typhus	Tick	Wild animals, cattle	Russia, Mongolia
	R. conori	Fever boutonneuse	Tick	Dog, rodents	Mediterranean
	R. conori	South African tick typhus	Tick	Dog, rodents	South Africa
	R. conori	Kenyan tick typhus	Tick	Rodents	Kenya
	R. conori	Indian tick typhus	Tick	Possibly rodents	India
	R. australis	Queensland tick typhus	Tick	Bush rodents	Australia
	R. japonica	Oriental spotted fever	Tick	Dog	Japan
	R. akari	Rickettsialpox	Gamasid mite	Mouse	USA, Russia
Scrub	*O. tsutsugamushi*	Scrub typhus	Trombiculid	Small rodents,	Asia, Pacific

1. Epidemic Typhus (Louse-Borne Typhus)

Caused by *R. prowazekii*, this infection is endemic in Eastern Europe, Africa, South America and Asia. **In India, the endemic spot is Kashmir. Humans are the only natural vertebrate hosts.**

> Natural infection in flying squirrels has been reported from southeastern USA. They may act as reservoir hosts, infection being spread by the squirrel louse and flea.

The human body louse *Pediculus humanus corporis* is the vector. The head louse may also transmit the infection but not the pubic louse. The rickettsiae multiply in the gut of the lice and appear in their feces in 3–5 days. Lice remain infective till they die—in 2 weeks. Being sensitive to temperature changes in the host, they leave the febrile patient to parasitise other persons. Infection is transmitted when contaminated louse feces enters the body through the minute abrasions caused by scratching.

The **incubation period** is 5–15 days. The disease starts with fever and chills followed by a characteristic rash on the fourth or fifth day on the trunk and limbs but not the face, palms and soles. Towards the second week, the patient becomes stuporous and delirious. Case fatality may reach 40 per cent and increases with age.

2. RECRUDESCENT TYPHUS (BRILL–ZINSSER DISEASE)

Epidemic typhus may remain latent and, at times, be reactivated, leading to **recrudescent typhus**. Such cases in louse-ridden communities may initiate epidemics of typhus fever.

3. Endemic Typhus (Flea-Borne Typhus)

R. typhi (*R. mooseri*) causes murine (or flea-borne) typhus, which is worldwide in distribution. While rats are the reservoirs, the infection is transmitted by the flea *Xenopsylla cheopis*. The *Rickettsia* multiplies in the gut of the flea and is shed in its feces.

Humans usually acquire the disease through:

- The bite of infected fleas, when their saliva or feces is rubbed in

- Through aerosols of dried feces
- By ingesting food recently contaminated with infected rat urine or flea feces

Human infection is a dead end. Person-to-person transmission does not occur. In Kashmir and China, lice have been known to transmit endemic typhus in humans, producing smouldering outbreaks.

Neil–Mooser reaction *R. typhi* and *R. prowazekii* are similar but may be differentiated by biological and immunological tests. When male guinea pigs are inoculated intraperitoneally with blood from a case of endemic typhus or with a culture of *R. typhi,* they develop **fever** and a characteristic **scrotal inflammation** (Neil–Mooser or tunica reaction). This reaction is negative with *R. prowazekii.* Other methods used include IFA, ELISA and PCR-based DNA tests.

Spotted Fever Group

1. Tick Typhus

The rickettsiae are transmitted transovarially in ticks, which, therefore, act as both vectors and reservoirs. Transmission to human beings is primarily by bite, as the rickettsiae also invade the salivary glands of the ticks. All rickettsiae of this group pass through natural cycles in domestic and wild animals or birds.

Rocky Mountain spotted fever is the most serious type of spotted fever. It is prevalent in many parts of North and South America and is transmitted by *Dermacentor andersoni* and related species of ticks. **Rashes** usually develop 2–4 days after the onset of fever (Fig. 38.3).

Fig. 38.3 Rocky mountain spotted fever (typical rashes on the right side of the face)

caused by *Rickettsia rickettsii* (*Source:* Public Health Image Library, ID 14489 / CDC)

R. conori causes **Indian tick typhus**. The disease was first observed in India by Megaw (1917) in the foothills of the Himalayas. The disease is found in many parts of India. The tick *Rhipicephalus sanguineus* is the most important vector. *Haemaphysalis leachi, Amblyomma* and *Hyalomma* ticks can also transmit the infection.

2. Rickettsialpox

The mildest rickettsial disease of humans is a self-limited, non-fatal, vesicular exanthema. It resembles chickenpox. It is also called vesicular or varicelliform rickettsiosis. The causative agent is *R. akari* (from *akari*, meaning mite). The **reservoir of the infection is the domestic mouse, while its vector is the mite** *(Liponyssoides sanguineus*, formerly, *Allodermanyssus),* in which transovarial transmission occurs. *R. akari* has also been isolated from wild rodents in Korea. The disease has also been reported in Eastern Europe and Korea.

GENUS ORIENTIA

Scrub Typhus (Chigger-Borne Typhus)

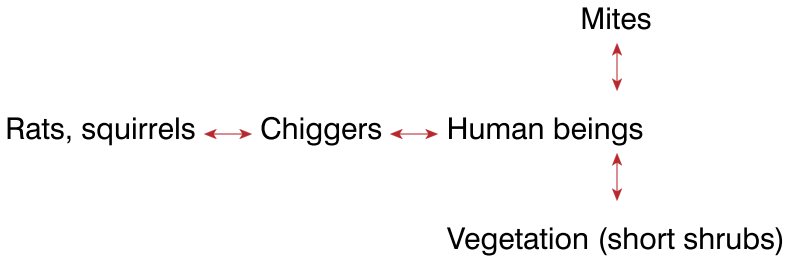
Scrub typhus is caused by *Orientia tsutsugamushi* (formerly known as *R. tsutsugamushi* and *R. orientalis*), an obligate intracellular parasite. This is an acute, febrile, infectious illness that was first described in China in 313 AD. *O. tsutsugamushi* occurs all along east Asia, from Korea to Indonesia, and in the Pacific Islands including Australia. It is found only in areas with a suitable climate, plenty of moisture and scrub vegetation. **Tsutsugamushi Triangle** refers to the region covering the Russian Far East in the north, Japan in the east, northern Australia in the south, and Afghanistan in the west. It is estimated that one million infections occur annually in this geographic region.

In India, scrub typhus has been steadily increasing and is reported from almost all the states in the last few years. Epidemics have been reported from Pondicherry, Andhra Pradesh and other southern states, Uttarakhand, and other states in North India. Urbanisation and increased diversion of forest land for agricultural use and climate change have impacted the

epidemiology, impacting the disease transmission, which is showing an upward trend. Availability of better tests has also resulted in increasing reports. In recent years, the epidemiological and demographic data of scrub typhus is being collected through the Integrated Disease Surveillance Programme (IDSP) of the Govt. of India.

Zoonotic tetrad

This is the co-existence and intimate relationship that exists among *O. tsutsugamushi*, chiggers, rats and secondary or transitional forms of vegetation. **In India, the vectors are trombiculid mites of the genus *Leptotrombidium deliensis***. These mites inhabit the soil and shrubs or bushes (low bushy trees with their woody branches close to the ground) where the microecosystem is favourable (**mite islands**). Humans are infected when they trespass onto these mite islands and are bitten by the **mite larvae (chiggers)**. These larvae seek moist areas of the body like the groin and axilla. They bite and cause **eschars**, a pathognomonic finding in scrub typhus. Adult mites, on the other hand, feed only on plants. Transovarian transmission occurs in mites. Various rodents and birds act as reservoirs.



The **incubation period** is 1–3 weeks. Patients develop a characteristic **eschar** at the site of the chigger bite, with regional lymphadenopathy and maculopapular rash. The disease sets in with fever, headache, muscle pain, nausea and vomiting. Encephalitis and pneumonia may be seen in severe cases. Other common complications associated with scrub typhus are acute kidney injury, hepatitis, acute respiratory distress syndrome (ARDS), myocarditis and septic shock.

Five major antigenic types have been recognised, which are widely used as in serologic assays. They are Boryon, Gilliam, Karp, Kato and Kawazaki.

Diagnosis of scrub typhus is by detecting **specific IgM antibodies by ELISA** or the non-specific test, Weil–Felix. A rising titre is suggestive of

scrub typhus.

The treatment of choice is doxycycline (200 mg, single dose) or tetracycline (100 mg, 4 times a day for seven days). Azithromycin 500 mg single dose or Levofloxacin can also be given in mild cases; however, it is not effective in severe cases with complications. No vaccines are available for the prevention of scrub typhus.

GENUS EHRLICHIA

Ehrlichiae are small, gram-negative, obligate intracellular bacteria which have an affinity towards blood cells.

Pathogenicity

Three human infections caused by this group of organisms have been identified:

1. **Glandular fever**—the patient shows serological response against the agent of canine ehrlichiosis. The causative agent has been named *Ehrlichia sennetsu.*
2. **Human monocytic ehrlichiosis**—caused by *Ehrlichia chaffeensis*. It is transmitted by *Amblyomma* ticks. Deer and rodents are believed to be reservoir hosts. Human disease is associated with leucopenia, thrombocytopenia and elevated liver enzymes.
3. **Human granulocytosis anaplasmosis (HGA)—**caused by *Anaplasma phagocytophilum.* This is endemic in the northeastern states of the US. It is transmitted by Ixodes ticks. Dogs and rodents are important reservoirs. Unlike other rickettsiae, this bacteria affects the granulocytes rather than mononuclear cells. Clinically, it is indistinguishable from *Ehrlichia*. This infection was earlier known as human granulocytic ehrlichiosis and the organism as *Ehrlichia equi.* The infection is treated with doxycycline.

LABORATORY DIAGNOSIS OF RICKETTSIAL INFECTIONS

Rickettsial infections are diagnosed in the laboratory mainly by serology.

Isolation by culture of rickettsiae is done in laboratories equipped with appropriate safety provisions (biosafety class III cabinets), as these

organisms are highly infectious. For this reason, isolation is not used as a routine diagnostic technique.

1. **Specimens** For diagnosis, blood and tissue samples are collected for culture, and serum for serological tests.

2. **Direct microscopy** It is possible to detect aggregations of rickettsial particles (but not individual particles) in cytoplasmic inclusion bodies under the light microscope by staining them with Giemsa (purple-coloured basophilic inclusions) or Machiavello stain (red-coloured inclusions).

Skin biopsies from the centre of the petechial lesions can be examined (up to 48 hours after anti-rickettsial drugs have been given) by one of the following methods:

- Immunofluorescence
- Immunoenzyme methods
- Immunohistochemical methods

3. **Culture** Cell and **tissue culture** are most widely used for isolation in containment laboratories. *Rickettsiae* grow well in 3–5 days on Vero cell or MRC 5 cell coverslip cultures, identified by immunofluorescence using group- and strain-specific antisera. Yolk sac of embryonated hens' eggs can also be used to isolate rickettsiae.

4. **Serological tests** Serological diagnosis is based on:

i) Detecting specific antibodies (generally IgM in acute infections) to rickettsial antigens by:

– **Immunoassays**

- Enzyme immunoassay (EIA/ELISA)
- Immunofluorescence
- Agglutination of rickettsial suspensions
- Passive hemagglutination of red cells sensitised by ESS (erythrocyte sensitising substance)

– **Complement fixation test** This is done with group-specific soluble antigen or the type-specific washed rickettsial antigen.

ii) **Weil–Felix reaction (non-specific test)** This is a test against agglutinins to the O antigens of certain non-motile *Proteus* strains: OX 19, OX 2 and OX K. The basis of the test is the sharing of an alkali-stable carbohydrate antigen by some rickettsiae and by *P. vulgaris* OX 19 and OX 2 and *P. mirabilis* OX K. The interpretations are given in Table 38.2.

Table 38.2 Weil–Felix reaction in rickettsial diseases

Disease	Agglutination pattern with		
	OX 19	*OX 2*	*OX K*
Epidemic typhus	+++	+	–
Brill–Zinsser disease	Usually negative or weakly positive		–
Endemic typhus	+++	±	–
Tick-borne spotted fever	++	++	–
Scrub typhus	–	–	+++

The antibody appears rapidly during the course of the disease, reaches peak titres of up to 1:1,000 or 1:5,000 by the second week and declines rapidly during convalescence. False positive reaction may occur in some cases of urinary or other infections by *Proteus* and at times, in typhoid fever and liver diseases. Hence it is desirable to demonstrate a rise in titre of antibodies for the diagnosis of rickettsial infection.

5. **Molecular methods** Rickettsial DNA can be detected by polymerase chain reaction, which permits rapid, specific identification of the infecting agent in skin biopsy, necrotic tissue and blood mononuclear cells.

Treatment of Rickettsial Infections

Rickettsiae are susceptible to doxycycline (the drug of choice for most rickettsial infections), tetracycline, chloramphenicol and ciprofloxacin. Penicillin and sulphonamides are ineffective. In fact, sulphonamides are contraindicated as they worsen the condition by enhancing the growth of

rickettsiae.

Immunoprophylaxis of Rickettsial Infections

There is no satisfactory vaccine available against any of the rickettsial diseases although historically, several killed and live vaccines have been tried.

Control of Rickettsial Infections

Eradication of rickettsial diseases is virtually impossible because of the cycles maintained in rodents, wild animals and vectors. Measures to reduce rodent or ectoparasite populations may help. Infested persons should be deloused, and their clothing and bedding decontaminated. Persons entering endemic areas should wear protective clothing to avoid infestation by vectors.

GENUS COXIELLA

Coxiella burnetii, the causative agent of Q fever, is a pleomorphic coccobacillary bacterium with a gram-negative cell wall and an ill-defined developmental cycle. **It is an obligate intracellular pathogen**, primarily infecting monocyte-macrophage cells. It occurs as rods, 0.2–0.4 × 0.4–1.0 μm in size or as spheres, 0.3–0.4 μm in diameter. It is filterable. Generally regarded as gram-negative, it is better stained with Gimenez and other rickettsial stains.

It shares many **common features** with the rickettsiae like:

- It exhibits obligate intracellular parasitism
- It has a cell wall composed of peptidoglycan
- It possesses both DNA and RNA
- It is susceptible to antibacterial agents

It **differs** in the following manners:

- By not having a vector for transmission.
- The Weil–Felix test **cannot** be used for the diagnosis of *Coxiella* infection.

- Infection is endemic and largely subclinical in cattle, sheep and goats, from where most human infection is acquired.

Antigenic phase variation *C. burnetii* shows phase variation. Fresh isolates are in Phase I. It becomes Phase II on repeated passage in the yolk sac but reverts to Phase I by passaging in guinea pigs. Phase I is a more powerful immunogen than Phase II and elicits good antibody response to both I and II antigens. Phase II antigen is more suitable for complement fixation tests.

Epidemiology

Q fever is distributed worldwide as a **zoonosis** acquired from domestic livestock. Wild animals such as the bandicoot may be the primary reservoir.

- It is transmitted among cattle, sheep and poultry by *Ixodid* ticks.
- Transovarial transmission occurs in ticks.
- *Coxiella* are abundant in tick feces and survive in dried feces for long periods.
- They are shed in the milk of infected animals.
- They are particularly abundant in their products of conception and contaminate the environment at parturition.

Pathogenicity

Human infections occur occupationally through the handling of wool or hides, meat or other animal products contaminated with the organism **(not by the bite of ticks**). Drinking infected milk can transmit the infection. ***Coxiella* may enter through abraded skin, mucosa, lungs** or the **intestinal tract**. Person-to-person transmission is rare.

C. burnetii is widely prevalent in birds and animals in India, as shown by serological surveys, but human disease has been identified only rarely.

The human disease is an acute systemic infection characterised by **interstitial pneumonia.** The clinical picture is very variable and asymptomatic infections very common.

In chronic Q fever, the organism spreads to almost all organs and may cause **hepatitis, meningoencephalitis or endocarditis**. Spontaneous

recovery is usual.

Coxiella may remain latent in the tissues of patients for 2–3 years**.**

Laboratory Diagnosis

- The diagnosis of Q fever is based mainly on **serological tests** using Phase I and II antigens using:
  - microagglutination,
    – complement fixation,
    – immunofluorescence and
    – enzyme-linked immunosorbent assay.
- PCR has been found to improve the early diagnosis of acute Q fever in the first four weeks of the disease, and a real-time PCR assay can also assess antibiotic susceptibilities.
- **Culture is not recommended due to the hazard of laboratory-acquired infection**.

Treatment

The treatment of choice is with tetracycline. Erythromycin, ciprofloxacin, rifampicin and the newer macrolides (clarithromycin) are also active. Treatment of endocarditis requires prolonged therapy, preferably with a combination of antibiotics (including tetracycline) for the first 14 days.

Immunoprophylaxis

A Q fever vaccine (Q-Vax) has been licensed for use in Australia and has effectively reduced the disease burden. Occupational exposure to high-risk animals or animal products poses a threat of transmission of Q fever to persons working in these areas. The vaccine is recommended for people aged 15 years and above who work in these high-risk occupations.

Vaccines have been prepared from formalin-killed whole cells, trichloracetic acid extracts and attenuated strains but they are not for general use.

ARTHROPOD-BORNE BACTERIAL INFECTIONS

BARTONELLA BACILLIFORMIS

Oroya fever (also known as Carrion's disease) caused by *B. bacilliformis* is seen inside erythrocytes and in skin lesions. It is a pleomorphic, gram-negative rod, which is motile by a tuft of polar flagella. It can be cultivated in semi-solid agar with rabbit or human blood The disease presents as fever and progressive anemia due to bacterial invasion of erythrocytes. Mortality is high in untreated cases. A late sequel in survivors or in those with asymptomatic infection is **verruga peruana**.

BARTONELLA (ROCHALIMAEA) QUINTANA

During the First World War, over a million cases of a disease known as **trench fever** or **five-day fever** occurred among soldiers fighting in the trenches in Europe (from *quintana*, meaning fifth, referring to 'five-day fever'.

Trench Fever

The causative agent is *R. quintana*. It is an exclusively human disease and **no animal reservoir is known**. It is transmitted by the body louse. The feces of lice become infectious 5–10 days after an infectious meal. The lice are unharmed and remain infective throughout their lives. Vertical transmission does not occur in lice. The disease frequently leads to chronic or latent infection. Recrudescence may occur as in Brill–Zinsser disease and relapses have been reported as long as 20 years after the primary disease.

The chronic infection and late relapses help to maintain the *Bartonella* in the absence of animal reservoirs.

Recent isolation of *B. quintana* from Tunisia and Mexico suggests that the disease may be more widely distributed. Trench fever cases have been identified in some homeless persons living in unsanitary conditions in the USA.

BARTONELLA HENSELAE

B. henselae has been linked to **'cat scratch disease'**, a febrile illness with lymphadenopathy following a cat scratch. There are two other conditions, seen more commonly in HIV-infected and other immunodeficient persons.

These are **bacillary angiomatosis,** in which vascular nodules or tumours appear on the skin, mucosa and other locations, and **bacillary peliosis** involving the liver and spleen. *B. henselae* can be demonstrated in lymph node biopsy smears and sections by Warthin–Starry staining.

Key points

- The family Rickettsiaceae comprises two genera: *Rickettsia* and *Orientia*.
- The family Anaplasmatacea comprises four genera: *Ehrlichia, Wolbachia, Anaplasma* and *Neorickettsia*.
- They share the common features of intracellular growth and transmission by hemagogous (blood-sucking) arthropod vectors (lice, fleas, ticks and mites).
- Rickettsiae are bacteria that cannot be seen by the ordinary light microscope and are obligate intracellular parasites. The important species in the family include *Rickettsia prowazekii, Rickettsia typhi and Rickettsia rickettsii.*
- In rickettsial infections, there is no direct human-to-human spread. Spread is from humans or an animal reservoir to an arthropod vector (lice, fleas, ticks and mites).
- Diagnosis by direct microscopy depends on the presence of cytoplasmic inclusion bodies.
- These organisms can be cultured in the yolk sac of embryonated hens' eggs, in mice, guinea pigs or in cell and tissue culture.
- Rickettsiae can be visualised microscopically in skin biopsies by immunofluorescence, immunoenzyme and immunohistochemical methods.
- The Weil–Felix test where certain antigens (OX19, OX 2, OXK) of *Proteus* are used is the most important diagnostic method.
- Other older serological tests include complement fixation and neutralisation.
- Rickettsial DNA can be detected by polymerase chain reaction.
- For treatment, tetracycline and chloramphenicol can be administered.
- Scrub typhus is caused by *Orientia tsutsugamushi*, which is transmitted by the bite of mite larvae (chiggers).
- Ehrlichiae are small, gram-negative, obligate intracellular bacteria which have an affinity towards blood cells.
- Doxycycline is recommended for the treatment of ehrlichiosis.
- *Coxiella burnetii* is a pleomorphic coccobacillary bacterium, which is a gram-negative obligate intracellular parasite. There is no vector for its transmission. The Weil–Felix test cannot be used for the diagnosis of *Coxiella* infection.
- Q fever is a worldwide zoonosis. Humans acquire the infection from animals among whom the disease is endemic and by inhalation of infectious aerosols.

- *Bartonella* are transmitted by arthropods and invade mammalian endothelial cells and blood cells.
- *B. bacilliformis* causes Oroya fever and verruga peruana; *B. quintana* causes trench fever and *B. henselae* causes 'cat scratch disease'.

Questions

Essay

Describe the pathogenesis and laboratory diagnosis of endemic typhus.

Short Answer

1. Q fever
2. Weil–Felix reaction
3. Scrub typhus
4. Trench fever

CHAPTER 39

Miscellaneous Bacteria Causing Human Infections

Chapter navigation

- *Yersinia*
- *Pasteurella*
- *Francisella*
- *Alcaligenes*
- *Flavobacterium*
- *Klebsiella* (*Calymmatobacterium*) *granulomatis*
- *Streptobacillus* and *Spirillum*
- Campylobacter
- *Helicobacter*
- *Legionella*
- *Capnocytophaga*
- *Gardnerella vaginalis*
- *Moraxella* (*Branhamella*) *catarrhalis*
- *Listeria*
- *Erysipelothrix*

INTRODUCTION

This group of bacteria do not belong to any single category of family or genus. However, they are human pathogens affecting several systems such as the central nervous, cardiovascular, respiratory and gastrointestinal systems. Infections can affect all ages and can be mild (*Helicobacter*) to life-threatening (*Yersinia*). Some of these organisms may be found in the environment causing opportunistic infections (*Legionella*) while others may be commensals.

YERSINIA, PASTEURELLA AND FRANCISELLA

These gram-negative, short bacilli that are primary pathogens of rodents

have been divided into three genera: *Yersinia*, *Pasteurella* and *Francisella*.

YERSINIA PESTIS

The most medically important species is *Y. pestis* (the causative agent of plague). The plague bacillus was discovered independently and simultaneously by Yersin and Kitasato (1894) in Hong Kong at the beginning of the last pandemic of the disease. It was known as **black death** in the middle ages.

Clinical Case 1

A 45-year-old farmer from a village was brought with septic shock and features of pneumonia to the emergency department of a nearby referral hospital. Relatives informed the attending doctor that the farmer had a sudden onset of fever with difficulty in breathing about two days earlier and hemoptysis subsequently.
X-ray at admission showed extensive bronchopneumonia. Immediate supportive therapy was started with antibiotics gentamicin and doxycycline. However, the patient succumbed to the infection.

On suspicion of plague, culture of the sputum and blood was performed in a containment (biosafety level III) referral laboratory. His sputum on Wayson stain showed bipolar staining bacilli, confirmed as Yersinia pestis *on culture. While this patient was being treated, a close household contact also developed similar symptoms. He was immediately started on doxycycline with supportive therapy and recovered. The incidences were notified to the public health authorities for immediate control programme.*

Morphology

Y. pestis is a short, plump, ovoid, gram-negative bacillus about 1.5 × 0.7 μm in size, arranged singly, in short chains or in small groups. Giemsa or methylene blue stain shows bipolar staining (**safety pin appearance**) with the two ends densely stained and a clear central area (Fig. 39.1a). Pleomorphism is very common, which is characteristically enhanced in media containing 3% NaCl. The bacillus is surrounded by a slime layer (envelope or capsule). It is non-motile, non-sporing and non-acid-fast.

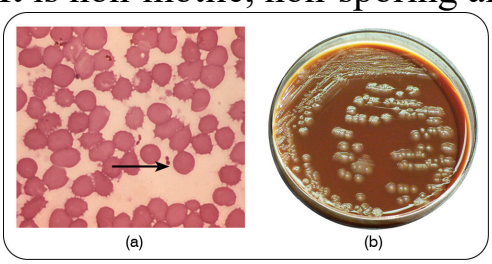


Fig. 39.1 (a) Smear of *Y. pestis* in blood smear showing safety pin appearance (bipolar staining) and (b) *Yersinia pestis* **bacteria, which was grown on a medium of chocolate**

agar (*Source:* (a) Public Health Image Library, ID 14555 / Dr. Jack Poland / CDC and (b) Public Health Image Library, ID 12472 / Amanda Moore, MT; Todd Parker, PhD; Audra Marsh / CDC)

Antigens, Toxins and Other Virulence Factors

Plague bacilli are antigenically homogeneous and serotypes do not exist. At least 20 antigens have been detected by gel diffusion and biochemical analysis. They are:

- Protein envelope antigen, **Fraction I** or **F-I,** which is heat-labile and is formed in cultures incubated at 37°C. **It inhibits phagocytosis and is generally present only in virulent strains**. This plasmid-encoded antigen has been considered a virulence determinant, but occasional strains deficient in the F-I antigen have been isolated from fatal human cases. The antibody to this antigen is protective in mice.
- **V** and **W** antigens, which are always produced together, have been considered to be virulence factors as they inhibit phagocytosis and intracellular killing of the bacillus. Production of these antigens is plasmid-mediated.
- **Pesticin I (bacteriocin) coagulase** and **fibrinolysin** are produced by virulent strains.
- **Plague toxin** refers to at least two classes of toxins found in culture filtrates or cell lysates:
    - **Endotoxin**, a lipopolysaccharide similar to the endotoxins of enteric bacilli
    - **Murine toxins,** the role of which in humans is not known
- **Unidentified surface component,** which absorbs hemin and basic aromatic dyes in culture media to form coloured colonies.
- Virulence is also associated with the ability to synthesise **purine**.

Resistance

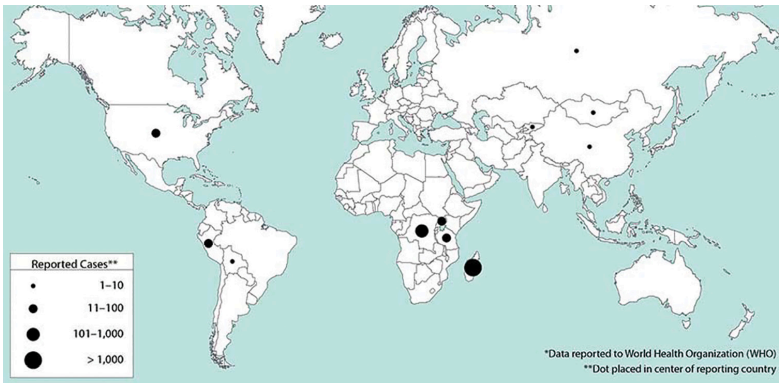
Y. pestis is easily destroyed by heat, desiccation and chemical disinfectants. It is destroyed at 55°C or by 0.5% phenol in 15 minutes. It remains viable for long periods in cold, moist environments. **It can survive for several**

months and even multiply in the soil of rodent burrows. All strains are lysed by a specific anti-*Yersinia pestis* bacteriophage at 22°C.

Plague

Plague is an ancient disease of humans. Central Asia, in particular the Himalayas, is the original home of the plague. It spread from there, causing epidemics and pandemics. In the fourteenth century, pandemic plague known as the **'black death'** was caused by *Y. pestis* var *medievalis.* It is believed to have killed a quarter of all humans.

The disease was quiescent in the eighteenth and nineteenth centuries and confined to endemic foci**.** The last pandemic which started in Hong Kong in 1894 and spread throughout the world was caused by *Y. pestis* var *orientalis*. Foci still exist in certain parts of the world (Fig. 39.2).



Reported* Plague Cases by Country, 2010–2015

Fig. 39.2 World distribution of plague, 2010–2015 (*Source:* https://www.cdc.gov/plague/maps)

Indian Scenario

Historical perspective in India

India was one of the countries worst hit by the plague pandemic. Plague reached Bombay in 1896 and spread all over the country during the next few years, causing more than 10 million deaths by 1918. It gradually receded thereafter, though occasional cases continued to be reported in endemic foci until 1967. No further plague cases were seen in India until 1994.

A non-fatal outbreak of bubonic plague was reported in 1994 in Maharashtra (Beed district). Later that year, pneumonic plague was reported in Surat and adjoining areas of Gujarat and Maharashtra, causing much panic and consternation. A few cases were reported from different parts of

North India as well, probably caused by the exodus of people from the affected areas. During the outbreak, which subsided in two months, there were over 6,000 suspected plague cases and 60 deaths. In February 2002, plague struck again, causing a short outbreak near Simla, which claimed four lives.

Four foci of plague are known in India:

i) The region near Kolar at the trijunction of Tamil Nadu, Andhra Pradesh and Karnataka.
ii) Beed–Latur belt in Maharashtra from where the Surat epidemic emanated.
iii) Rhoru in Himachal Pradesh where the 2002 outbreak took place.
iv) A small pocket in Uttaranchal.

Types of Plague

In human beings, plague occurs in three main forms:
(i) bubonic, (ii) pneumonic and (iii) septicemic.

Bubonic plague

The incubation period is 2–5 days after the bite of an infected flea. Draining lymph nodes of the site of entry of the bacillus become infected (Fig. 39.3a). In some cases, only minor constitutional symptoms (pestis minor) occur. Commonly, the flea bites on the legs, hence the inguinal nodes are involved, giving the disease its name, 'bubonic' (*bubon* meaning groin) plague. The glands enlarge and suppurate. The bacilli enter the bloodstream and produce septicemia. Sometimes, there are hemorrhages into the skin and mucosa. **Disseminated intravascular coagulation** is common and may lead to **gangrene** of the skin, fingers and penis (Fig. 39.3b). Case fatality in untreated cases may be 30–90 per cent.

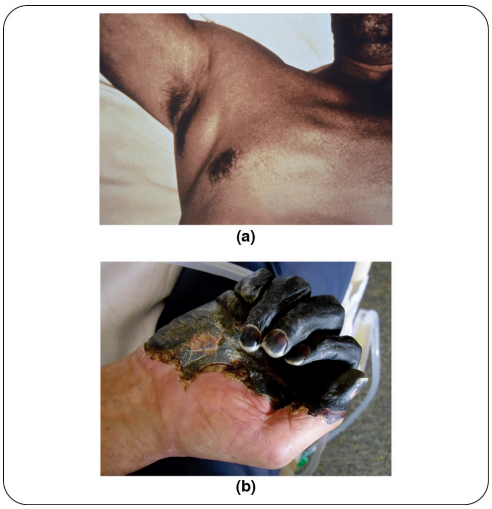


Fig. 39.3 Characteristic features of plague: (a) swollen axillary lymph node (Bubo) and (b) gangrenous condition of the fingers and palm due to *Yersinia pestis* infection (*Source:* (a) Public Health Image Library, ID 2045 / CDC and (b) Public Health Image Library, ID 16551 / Christina Nelson, MD, MPH / CDC)

Pneumonic plague

This type may sometimes be seen during epidemics of bubonic plague. Rarely, primary pneumonic plague may occur in epidemic form, as happened in Manchuria in 1910–1912, causing some 60,000 deaths. Pneumonic plague is spread by droplet infection. The bacilli spread through the lungs, producing hemorrhagic pneumonia. Cyanosis is very prominent. The bloody mucoid sputum that is coughed out contains bacilli in enormous numbers. Pneumonic plague is highly infectious and in untreated patients, almost invariably fatal (*Clinical case* 1).

Septicemic plague

This is usually the terminal event in the bubonic or pneumonic plague but may sometimes occur primarily. Meningitic involvement may occur rarely. Human carriers have not been recorded but asymptomatic oropharyngeal infection has been observed in some contacts.

Epidemiology

Plague is a zoonotic disease. The plague bacillus is naturally parasitic in rodents. Infection is transmitted among them by rat fleas. The fleas acquire the infection by feeding on infected rodents. In the flea, the bacilli multiply in the stomach to such an extent that they block the proventriculus. The

interval between the ingestion of infected blood and blocking in the proventriculus is the **extrinsic incubation period**, which is usually about two weeks in *Xenopsylla cheopis* (Fig. 39.4a). When such a 'blocked' flea bites another rodent, it cannot suck in blood because the bacterial mass blocks the passage mechanically. Blood, mixed with the bacteria, is regurgitated into the bite, transmitting the infection. Infection may also be transferred by the contamination of the bite wound with the feces of infected fleas. When a diseased rat dies (rat fall), the fleas leave the carcass and, in the absence of another rat, may bite human beings (Fig. 39.4b), causing bubonic plague.

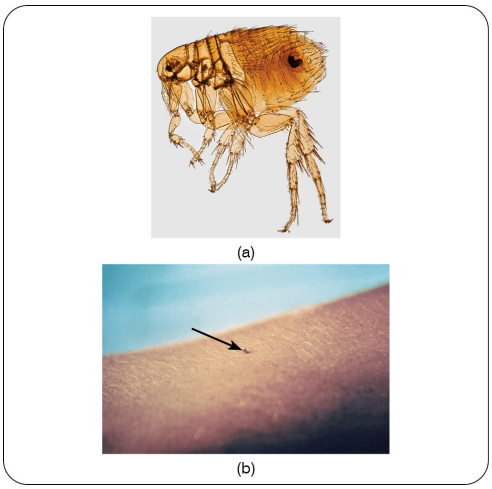


Fig. 39.4 (a) Oriental rat flea, *Xenopsylla cheopis*, a well-known bubonic plague vector and (b) the **flea biting its human host** (*Source:* (a) Public Health Image Library, ID 22259 / Ken Gage / CDC and (b) Public Health Image Library, ID 14286 / CDC)

***X. cheopis*, the predominant species in North India,** is a more efficient vector than the South Indian species *X. astia.* It has caused extensive nature of plague outbreaks in the North as compared to those in South India. Transmission in cool climate depends on high **'flea index'** (mean number of fleas per rat). The Plague Commission in Mumbai found that plague produced epizootics first in *Rattus norvegicus* (sewer rat). When their number dwindled, the disease passed to the domestic rat, *R. rattus.* **It was from the domestic rat that the infection spread to human beings.**

Two natural cycles of plague exist, the domestic and the wild:

- '**Urban or domestic plague**' refers to plague that is associated with human beings and the rodents living around them.

- '**Wild or sylvatic plague**' occurs in nature and in wild rodents, independent of human beings. **In India, the gerbil (*Tatera indica*) and the bandicoot are also infected.** Human infection may occur during skinning and handling of carcasses of infected wild animals.

Carnivores, including cats and dogs, can get infected by eating infected rodents or through their fleas. Clinical plague is seldom seen in dogs but may develop in cats. Human infection from inhalation of respiratory droplets from infected cats has been reported.

In **enzootic foci**, plague may persist for long periods. Infected fleas may survive for over a year. The bacilli can remain alive and even multiply in the soil of abandoned rodent burrows. They can infect new rodents that may reoccupy such burrows. Only when human beings or domestic animals trespass on these natural foci do human infections set in on them.

In the 1990s, there was a re-emergence of plague in India and China in Asia, Malawi and Zimbabwe in Africa, in the erstwhile USSR, in Europe and in the USA.

Laboratory Diagnosis

Containment laboratories with biosafety levels III or IV are empowered to handle and process specimens from patients and rodents with plague.

In humans

1. **Specimen** Buboes, sputum and blood are collected under strict sterile conditions.
2. **Direct demonstration** In human bubonic plague, the bacilli may be readily demonstrated in buboes by microscopy, culture or animal inoculation. Blood cultures are often positive. In pneumonic plague, the bacilli can be demonstrated in sputum by microscopy, culture or animal inoculation.

Microscopically, smears from the bubo, sputum or blood stained with methylene blue (Wright or **Wayson stain**) show bipolar stained bacilli (Fig. 39.5).

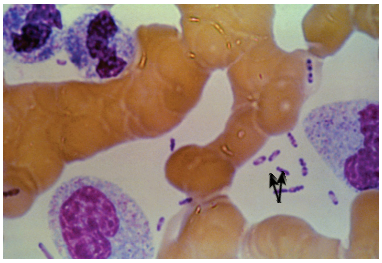


Fig. 39.5 Bubo smears in Wright stain showing bipolar staining (safety pin appearance) of *Y. pestis* in blood smear (*Source:* Public Health Image Library, ID 2050 / CDC)

Fluorescent antibody technique may be used to identify plague bacilli in the impression films of the tissues by demonstrating the F-I antigen. Bacilli in buboes show considerable pleomorphism.

Cultures may be made from the buboes, spleen, heart blood and particularly, from bone marrow of carcasses.

3. **Cultural characteristics** *Yersinia pestis* is aerobic and facultatively anaerobic. Growth occurs over a wide range of pH (pH 5–9.6, optimum pH 7.2) and temperature (range 2–45°C). Unlike most pathogens, **the optimum temperature for its growth is 27°C** but the envelope develops best at 37°C.

- Colonies on **blood agar or chocolate agar** or other hemin-containing media are dark brown due to the absorption of the hemin pigment (Fig. 39.1b).
- Colourless colonies are formed on **MacConkey agar**.
- In **broth**, a flocculent growth occurs at the bottom and along the sides of the tube and a delicate pellicle may form later. Within a medium with a film of oil or ghee (**ghee broth**), a characteristic growth occurs which hangs down into the broth from the surface, resembling stalactites (**stalactite growth**).

4. **Biochemical reactions** Glucose, maltose and mannitol (but not lactose, sucrose and rhamnose) are fermented with the production of acid but no gas. Indole is not produced. It is MR positive and VP- and citrate-negative, catalase- and esculin-positive, and oxidase- and urease-negative. Gelatin is not liquefied.

5. **Serological tests** Antibodies to the F-I antigen may be detected by

passive hemagglutination. Rise in titre of antibodies in paired sera or titre of 128 or above in a single serum sample can be considered positive. IgG and IgM ELISA tests and a rapid dipstick assay have now been developed using the F-I antigen.

6. **Molecular methods**

Clinical material and fleas PCR is a rapid and sensitive method for the presumptive diagnosis of plague.

Isolation from rats

A dead rat may carry infected fleas, hence should be handled with care. In the laboratory, the carcass is dipped in 3% Lysol to destroy the fleas.

Managing plague outbreaks (as per WHO guidelines)

- **Find and stop the source of infection.** Clustered areas with large numbers of small animal deaths are the first indication. First vector and then rodent control must be done. (Killing rodents before vectors will cause the fleas to jump to new hosts.)
- **Protect health workers**. Workers in direct contact with pneumonic plague patients must practice standard precautions and receive chemoprophylaxis with antibiotics for seven days or at least as long as they are exposed to infected patients.
- **Ensure correct treatment with appropriate antibiotics.**
- **Isolate patients with pneumonic plague**.
- **Obtain specimens** which should be carefully collected using appropriate infection prevention and control procedures and sent to labs for testing.
- **Disinfection is very important.** Routine hand-washing is recommended with soap and water or alcohol hand rub. Larger areas can be disinfected using 10% solution of diluted household bleach (made fresh daily).
- **Surveillance** of close contacts of pneumonic plague patients should be done and they should be provided seven-day chemoprophylaxis. Cotrimoxazole or tetracycline is given orally.
- **Safe burial practices should be followed.** The area should be covered with a disinfectant-soaked cloth or absorbent material.

(Reference: https://www.who.int/fact-sheets/detail/plague)

Prophylaxis

General measures Control of fleas and rodents is of paramount importance.

Vaccines Two types of **vaccines** have been in use:

i) **Whole-culture antigen** is a **killed vaccine used in India (prepared at the Haffkine Institute, Mumbai**). The vaccine is given subcutaneously, in two doses at an interval of 1–3 months, followed by a third, six months later.

To prepare this vaccine, a virulent strain of the plague bacillus is grown in casein hydrolysate broth

for 2–4 weeks at 32°C and killed by 0.05% formaldehyde and preserved with phenylmercuric nitrate.

Vaccination gives some protection against bubonic plague but not against pneumonic plague. Protection does not last for more than six months.

ii) **A recombinant rF1V plague vaccine** has been developed by scientists at the US Army Medical Research Institute of Infectious Diseases (USAMRIID). The vaccine is stated to be used for pre-exposure prophylaxis in plague.

WHO recommends vaccination only for high-risk groups (only for those exposed occupationally or otherwise to infection, such as a plague laboratory or hospital personnel and troops deployed in known plague areas). It has no value in outbreaks or mass vaccination.

An attack of plague confers life-long immunity.

Chemoprophylaxis A person exposed to a definite risk of infection, whether vaccinated or not, should be given chemoprophylaxis—cotrimoxazole or tetracycline orally for seven days.

Treatment

Early treatment with antibiotics has reduced plague mortality from 30–100 per cent to 5–10 per cent. Streptomycin, doxycycline and chloramphenicol are effective in treatment. Plasmid-borne resistance to multiple antibiotics was reported in 1995 from Madagascar. These strains have the potential to spread and pose a global threat.

Yersiniosis

The term yersiniosis denotes infections with yersiniae other than *Y. pestis*. These include zoonotic infections by *Y. enterocolitica* and *Y. pseudotuberculosis*.

***Yersinia pseudotuberculosis* It is a pathogen of guinea pigs and rodents.** It closely resembles *Y. pestis* but can be distinguished by its relatively poor growth on MacConkey agar, motility at 22°C (but not at 37°C), production of urease, fermentation of rhamnose and melibiose and failure to be lysed by the anti-*Y. pestis* bacteriophage at 22°C. Distinction between

Y. pseudotuberculosis and *Y. pestis* becomes important when the former is isolated from rats.

***Y. enterocolitica* is described in Chapter 30, as it is now considered under the family Enterobacteriaceae and is one of the enteric pathogens**.

PASTEURELLA MULTOCIDA

The genus *Pasteurella* occasionally produces local and systemic infections in human beings. It contains several related bacteria causing hemorrhagic septicemia in different species of animals. A common species is *P. multocida*.

> One of these, *P. aviseptica,* is the chicken cholera bacillus used by Pasteur for the development of the first attenuated bacterial vaccine; hence the name **Pasteurella.** The other species, *P. boviseptica* and *P. lepiseptica*, are mainly animal pathogens.

P. multocida is a non-motile, gram-negative bacillus generally resembling *Yersinia* but differing in being oxidase-positive, producing indole and failing to grow on MacConkey agar.

The bacillus is often carried in the upper respiratory tract of a variety of domestic animals and rodents. Human infection is rare but may occur following **animal bites** or trauma. The clinical manifestations may be **local suppuration** following animal bites (wound infection, cellulitis, abscess, osteomyelitis), **meningitis** following head injury, **respiratory tract infection** (pneumonia, bronchitis, sinusitis) or appendicitis and appendiceal abscess.

The bacillus is sensitive to tetracycline and streptomycin, and most strains to penicillin as well.

FRANCISELLA TULARENSIS

Francisella tularensis (after Dr Edward Francis for his work on tularemia originally described in Tulare County, California), is the causative agent of **tularemia,** a disease of rabbits and other rodents, originally. Infection is transmitted by ticks. Human infection may occur by direct contact with infected rodents such as rabbits or through tick bites. It can also be acquired by the ingestion of contaminated meat or water and inhalation of infective

aerosols.

It is a minute, capsulated, non-motile, gram-negative bacillus, about 0.3–0.7 × 0.2 μm in size. It is filterable and multiplies by filament formation and budding besides binary fission (resembling *Mycoplasma*). It is an intracellular parasite, found in large masses inside liver and spleen cells. It is fastidious and grows only on **Francis' blood dextrose cysteine.** Minute transparent colonies appear after incubation for 3–5 days. The bacillus is highly infectious and laboratory infection has been quite common.

Strains of *S. tularensis* have been subdivided into biotypes. Virulent strains are found only in North America, while strains of low virulence are seen in Europe and Asia as well.

In humans, tularemia may present as local ulceration with lymphadenitis, a typhoid-like fever with glandular enlargement or an influenza-like respiratory infection. Diagnosis may be made by culture or by inoculation into guinea pigs or mice.

Agglutinating antibodies may be demonstrated in sera from patients. An attenuated vaccine is available for administration by scarification to persons at high risk of infection.

ALCALIGENES FAECALIS

Alcaligenes faecalis is a gram-negative, short, non-sporing bacillus. It is a saprophyte found in water and soil contaminated with decaying organic matter. It can also be a commensal in humans. The bacilli can be isolated from the hospital environment in respirators, nebulisers, etc. They have been isolated from a variety of clinical specimens such as urine, pus and blood. *A. faecalis* is associated with urinary infections, infantile gastroenteritis and suppurative lesions.

CHROMOBACTERIUM VIOLACEUM

Chromobacterium violaceum is a gram-negative, non-sporing bacillus, motile by means of polar and lateral flagella, resembling pseudomonads. It is a facultative anaerobe, growing on ordinary media and producing a violet pigment soluble in ethanol and insoluble in water and chloroform. Human infections have been recorded mainly in the tropics, and consist of skin lesions with pyemia and multiple abscesses.

FLAVOBACTERIUM MENINGOSEPTICUM

Flavobacterium meningosepticum is a non-motile, gram-negative bacillus, producing a yellowish pigment. It is oxidase-positive, proteolytic and weakly fermentative. It is a ubiquitous saprophyte capable of causing opportunistic infections. It has been responsible for outbreaks of meningitis in newborn infants. Infection in adults leads to a mild febrile illness.

KLEBSIELLA GRANULOMATIS

It was formerly known as *Donovania granulomatis* and *Calymmatobacterium granulomatis*. *Klebsiella granulomatis* appears as rounded coccobacilli, 1–2 μm in size, within cystic spaces in large mononuclear cells
(Fig. 39.6a). They show bipolar condensation of chromatin, giving a closed safety pin appearance in stained smears. Capsules are usually seen as dense acidophilic areas around the bacilli. They are non-motile and gram-negative. They can be grown on egg yolk medium and on modified Levinthal's agar.

Fig. 39.6 (a) **Photomicrograph of a tissue sample extracted from a lesion in the inguinal region of the female granuloma inguinale shows a white blood cell (WBC) containing the pathognomonic finding of Donovan bodies and** (b) **large erosive cutaneous lesion in the perineal region diagnosed as donovanosis** (*Source:* (a) Public Health Image Library, ID 18899 / Susan Lindsley / CDC and (b) Public Health Image Library, ID 15433 / Joe Miller / CDC)

Pathogenicity

It is the causative agent of **donovanosis** or **granuloma inguinale**, one of the sexually transmitted infections. The incubation period ranges from 1 to 12 weeks. It begins as a painless papule on the genitalia, which leads to a slowly progressive, autoinoculable ulcer and runs a chronic course (Fig. 39.6b). Donovanosis is a venereal disease and its pathogenicity is limited to human beings.

Laboratory Diagnosis

A diagnosis of donovanosis can be made by the demonstration of **Donovan bodies** in Wright–Giemsa-stained impression smears from the lesions (Fig. 39.6a).

Treatment

Tetracycline given for at least three weeks is usually curative. Cotrimoxazole, chloramphenicol, gentamicin, quinolones and the newer macrolides are also effective.

STREPTOBACILLUS MONILIFORMIS AND SPIRILLUM MINUS

Two different bacteria—*Streptobacillus moniliformis* and *Spirillum minus*—both of which are natural parasites of rodents, are responsible for a disease called **rat bite fever (RBF)**. The disease is characterised by relapsing fever, rash and arthralgia occurring days or weeks after a rat bite.

S. moniliformis is a highly pleomorphic, gram-negative, non-motile bacillus. In cultures, it grows as tangled chains of rods of various lengths, with beaded or fusiform swellings, readily developing into L-forms. Infection is called **Haverhill fever** or **erythema arthriticum epidemicum**. It is believed to be also caused by the consumption of raw milk or water contaminated by rat droppings.

Laboratory diagnosis is by the isolation of the bacillus from blood or other body fluids. Smears of joint fluid may show pleomorphic, gram-negative rods. Agglutination, complement fixation and fluorescent antibody tests have been used for serological diagnosis.

Spirillum minus is a short, actively motile bacterium, 3–5 × 0.2–0.5 μm in size, with two or three regular spirals and 1–7 amphitrichous flagella. It is gram-negative but is better visualised by Giemsa or Fontana stains or by dark-field microscopy. Spirillary RBF has an incubation period of 1–4 weeks. The rat bite wound, which may have healed, suppurates at the onset of fever, with regional lymphadenopathy. The subsequent course is similar to the streptobacillary type. Endocarditis causes mortality in 10 per cent of cases.

Laboratory diagnosis is by the **microscopic examination** of blood and exudates from the lesion by intraperitoneal inoculation into guinea pigs and mice and by demonstration of the spirilla in their blood and peritoneal fluid.

Treatment

Both types of RBF respond to penicillin and tetracycline. Oral penicillin or doxycycline after a rat bite is effective in prophylaxis.

CAMPYLOBACTER

The genus *Campylobacter* (Greek, meaning 'curved rod') are slender, spirally curved, gram-negative rods, 0.2–0.5 μm thick and 0.5–5 μm long. They are typically comma-shaped but may occur as 'S' or multispiral chains. They are motile with a single unsheathed polar flagellum at one or both poles. Growth occurs under microaerophilic conditions, 5% oxygen concentration being optimal. Many pathogenic species are thermophilic, growing well at 42°C. Campylobacters do not ferment carbohydrates but are strongly oxidase-positive.

Resistance

C. jejuni, *C. coli* and *C. lari* are thermophilic and do not grow at 25°C. Plates are incubated at 42°C in an atmosphere of 5% oxygen, 10% carbon dioxide and 85% nitrogen.

Campylobacters first gained prominence in the 1970s as a common cause of human diarrheal disease, affecting children and adults. They can, on occasion, also cause systemic infections. They are important veterinary pathogens. Campylobacters of medical importance are as follows:

- Causing diarrheal disease: *C. jejuni, C. coli, C. lari*
- Causing extraintestinal infection: *C. fetus*
- Causing abscess: *C. sputorum, C. conciscus*

Medically, ***Campylobacter jejuni*** is the most important campylobacter species as it causes attacks of diarrhea worldwide. The infection is zoonotic, the source being food of animal origin, especially raw milk. It is part of the normal intestinal flora of domestic animals and birds and is shed in their feces. It can be isolated frequently from surface waters.

Pathogenicity

C. jejuni is the most common bacterial cause of **diarrheal disease** in many developed countries—more common than salmonellae or shigellae. *C. jejuni* is endemic in developing countries; asymptomatic infection is prevalent in humans as well as domestic animals and birds. The disease is mainly confined to children, while the older age groups are immune. Infection occurs by ingestion. The jejunum and ileum are the primary sites of colonisation but it may spread to the colon and rectum. *C. jejuni* is an invasive pathogen involving the mesenteric lymph nodes. The incubation period is 1–7 days. *Campylobacter* is thought to invade and damage cells of the small intestine, disrupting fluid absorption.

The illness starts with fever, abdominal pain and watery diarrhea. Stool contains leucocytes and blood. The disease is usually self-limiting, though *Campylobacter* shedding may continue for weeks after recovery.

C. coli causes an infection that is clinically indistinguishable from that caused by *C. jejuni. C. coli* is commonly found in healthy pigs. *C. lari*, differentiated from others by being resistant to nalidixic acid, also causes a similar diarrheal disease.

Laboratory Diagnosis

Laboratory diagnosis depends on the isolation of the campylobacter from feces.

Direct microscopic examination by phase-contrast or dark-field microscopy to detect the darting or tumbling motility of the spiral rods or **demonstration of the small curved rods in stained smears** may be useful

for presumptive rapid diagnosis.

Feces or rectal swabs are plated on selective media. Campylobacters survive for 1–2 weeks at 4°C in **Cary–Blair transport medium**. Media commonly used for culture are **Skirrow's**, **Butzler's** or **Campy BAP selective media**.

Colonies usually appear in 48 hours. They are non-hemolytic, grey or colourless, moist and flat or convex. They are confirmed by Gram stain, darting motility on wet mount and biochemical tests, the most common of which is a positive hippurate hydrolysis.

Treatment

Fluid and electrolyte replacement is all that is generally required. When needed, erythromycin is the best antibiotic.

Campylobacter fetus is the other species which may infect humans leading to bacteremia, sepsis and meningitis in immunocompromised hosts. The portal of entry is the gastrointestinal tract.

HELICOBACTER

Spiral, *Campylobacter*-like bacteria were observed in close apposition to the gastric mucosa in several cases of gastritis and peptic ulcer by Warren and Marshall in Australia in 1983. They were originally named *Campylobacter pylori* but later re-designated as *Helicobacter pylori*.

By enzyme immunoassay, *Helicobacter* antigens have been detected in the intestines of pre-Columbian mummies in the USA. Today, helicobacters colonise the stomachs of half the human population of the world!

Clinical Case 2

A 35-year-old man presented to the hospital complaining of pain in the epigastrium along with nausea, flatulence and bad breath for the previous four weeks. The pain typically increased in the middle of the night and decreased slightly after eating a meal. Antacids did not relieve the symptoms. On examination, there was epigastric tenderness.

An endoscopic examination followed by gastric biopsy was done. The tissue bit was positive for rapid urease test. Histopathology of the tissue showed the presence of gram-negative, spiral bacilli in the submucosa. A diagnosis of gastric ulcer with pylori gastritis was made. The patient was relieved of his symptoms after treatment with a combination of amoxicillin, clarithromycin and omeprazole along with other supportive care.

H. pylori is adapted to the human gastric mucosa. The only other animal it infects is the monkey. Other species of interest are: *H. heilmannii,* which can occasionally infect humans and some animals like cats and dogs and *H. cinaedi* and *H. fennelliae,* which are associated with proctitis in the HIV-infected individuals.

HELICOBACTER PYLORI

H. pylori is a gram-negative spiral rod, motile by a unipolar tuft of lophotrichous flagella. It grows on chocolate agar or *Campylobacter* media under microaerophilic conditions, with 5–20% CO_2 and pH 6–7. At 37°C, colonies take 2–7 days to develop. Coccoid forms appear in old cultures. The organism produces oxidase, catalase, phosphatase and H_2S.

A distinctive feature of *H. pylori* is the production of abundant urease, and this property has been used as a rapid urease test in gastric biopsy samples. It does not metabolise carbohydrates or reduce nitrate.

Virulence Factors

Certain virulence factors have been incriminated in the pathogenesis:

- Vacuolating cytotoxin A
- Cytotoxin-associated gene A
- Urease enzyme
- Outer membrane proteins
- Lipopolysaccharide

H. pylori shows considerable **genetic diversity**, as is evident in molecular typing. The complete genome of the bacterium has been mapped. Virulence has been associated with certain alleles in genes, such as ***cag*** (cytotoxin-associated gene) and ***vac*** (vacuolating cytotoxin gene). Urease production and motility also help in pathogenesis.

Epidemiology

H. pylori is global, with a prevalence of 30–60 per cent, higher in developing than in the developed countries. The sole source of *H. pylori* is

the human gastric mucus. The exact mechanism of transmission is not clear but it is likely to be oral–oral or fecal–oral. Poverty, overcrowding and poor hygiene favour transmission. With improvements in lifestyle, the prevalence of childhood infections has declined in the developed countries.

Pathogenicity

After an **incubation period** of a few days, *H. pylori* causes, in some persons, a mild acute gastritis which may last for about two weeks. Symptoms subside and the organism persists as an asymptomatic coloniser, though chronic superficial gastritis may be demonstrable histologically. The bacteria are present only in the overlying mucus and **do not invade the mucosa**; this protects the bacteria from the acidic pH of the stomach. Being strictly confined to the gastric mucosa, the **gastric antrum is the commonest site of colonisation**, though any part of the stomach may be involved. Infection leads to areas of gastric metaplasia and heterotopia in the duodenum.

Peptic ulcer disease occurs in a proportion of the infected (*Clinical case* 2). Chronic atrophic gastritis may be seen in the later stages. The **infection is recognised as a risk factor for gastric malignancies such as adenocarcinoma and 'mucosa-associated lymphoid tissue' (MALT) lymphomas**. Such MALTomas appear to be antigen-driven and are found to regress after the elimination of *H. pylori* by treatment. Infection induces IgM, IgG, IgA and cellular immune response but these do not seem to be protective.

Laboratory Diagnosis

Diagnostic tests are of two kinds:

1. **Invasive tests** (endoscopic biopsy of gastric mucosa):
   i) **The tissue is subjected to rapid urease test.**
   ii) Part of the tissue is sent for examination by microscopy and culture. Microscopy of biopsy sections by silver staining or of Gram-stained smears is a useful method and is positive for spiral bacilli (Fig. 39.7).

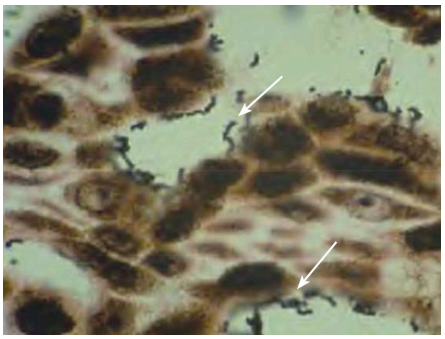


Fig. 39.7 *Helicobacter pylori* gastritis—silver stain

iii) Culture is more sensitive, requires enriched medium, in which the bacteria grows at 37°C in microaerophilic conditions and within 3–7 days. It is catalase- and oxidase-positive.

2. **Non-invasive tests** IgM and IgG detection by ELISA are no longer used as they are not helpful in the diagnosis. However, they may be used for seroepidemiological studies in the community.

Rapid tests Several rapid immunochromatographic cassette and dipstick methods have been developed for bedside testing for antigens or antibodies in stool, gastric aspirate or blood.

In the **'urease breath, test'**—the subject drinks a carbon isotope-labelled urea solution. This isotope can be detected in the breath. This method is sensitive and reliable but needs isotope assay facilities.

Treatment

Indications are an active peptic ulcer disease or past history of peptic ulcer without documented cure.

H. pylori is sensitive to several antibiotics and bismuth salts.

There are different treatment regimens. The standard treatment is a combination of bismuth subsalicylate, tetracycline (or amoxicillin) and metronidazole for two weeks.

- The first-line treatment consists of clarithromycin, amoxicillin and metronidazole—BID 14 days, clarithromycin (500 mg), amoxicillin (1 g) or metronidazole (500 mg TID).
- Bismuth, tetracycline and a nitroimidazole for 10–14 days is the second line of treatment.

An alternative schedule uses a proton pump inhibitor like omeprazole and clarithromycin. Treatment is indicated only for *H. pylori*-related gastric or duodenal ulceration and not for asymptomatic colonisation. Drug resistance and recurrence are frequent.

LEGIONELLA PNEUMOPHILA

The name Legionnaires' disease, which is caused by *Legionella pneumophila,* was given to an apparently new illness that broke out among members of the American Legion who attended a convention in Philadelphia in 1976. The disease was characterised by fever, cough and chest pain, leading to pneumonia and often ending fatally.

Morphology

Legionellae are thin, non-capsulated bacilli, 2–5 × 0.1–0.3 μm in size. They are coccobacillary, assuming longer forms in culture. Most are motile with polar or subpolar flagella. They are gram-negative but stain poorly. They stain better by silver impregnation but are best visualised by **direct fluorescent antibody (DFA**) staining with monoclonal or polyclonal sera.

Epidemiology

Legionellae are widely distributed in natural water sources such as stagnant waters, mud and hot springs where the nutritional and growth requirements for these fastidious bacteria are provided by some types of algae**. Legionellae survive and multiply inside free-living amoebae and other protozoa.** Human infection is by inhalation of aerosols produced by cooling towers, air conditioners and showerheads which act as disseminators. Infection is limited to human beings. No carrier state is established. **Human-to-human transmission does not occur.**

Known risk factors are smoking, alcohol, advanced age, intercurrent illness, hospitalisation and immunodeficiency. Men are more often affected than women. In the developed countries, legionellosis accounts for 1–3 per cent of community-acquired and 10–30 per cent of hospital-acquired pneumonias. Its prevalence in the developing countries is not adequately known. Two distinct clinical patterns have been identified and designated as:

i) **Legionnaires' disease** and
ii) **Pontiac fever**.

Together, they are known as **legionellosis**.

Pathogenicity

Following entry into the alveoli through aerosols, legionellae multiply inside the monocytes and macrophages. Dissemination occurs by endobronchial, hematogenous, lymphatic or contiguous spread. The incubation period is 2–10 days. There is fever, non-productive cough and dyspnea, rapidly progressing, if untreated, to pneumonia. Diarrhea and encephalopathy are common. Case fatality may be 15–20 per cent, the cause of death being progressive respiratory failure and shock. All age groups are susceptible, though more cases have occurred in the elderly. Because of their intracellular location, humoral antibodies are ineffective. Cellular immunity is responsible for recovery.

Pontiac fever is a milder, non-fatal, 'influenza-like' illness with fever, chills, myalgia and headache. Outbreaks with high attack rates may occur.

The original isolate in this genus is designated *L. pneumophila* serogroup 1 (SG1), which accounts for nearly all severe infections. Examples of other species that cause human infection less often are *L. micdadei, L. bozemanii, L. dumoffii* and *L. gormanii.*

Laboratory Diagnosis

Cultural characteristics Sputum, bronchial aspirates and lung biopsies are examined by direct fluorescent antibody test and culture.

They are fastidious organisms that grow on complex media—buffered charcoal yeast extract **(BCYE)** agar—with L-cysteine and antibiotic supplements, with 5% CO_2 at pH 6.9, temperature 35°C and 90% humidity. Growth is slow and colonies take 3–6 days to appear.

Legionella antigens in urine can be detected by latex agglutination. Alternatively, ELISA antibodies are detected by ELISA or indirect immunofluorescent assay.

Treatment

Newer macrolides, ciprofloxacin and tetracyclines are effective. Rifampicin

is used in severe cases. **Beta- lactamase antibiotics and aminoglycosides are ineffective.**

CAPNOCYTOPHAGA

The *Capnocytophaga* species are gram-negative, fusiform gliding bacilli which form part of the normal mouth flora. They may occasionally cause systemic infections in the immunodeficient.

GARDNERELLA VAGINALIS

Gardnerella vaginalis is a small, gram-negative, non-motile, pleomorphic rod which shows metachromatic granules. It was formerly known as *Haemophilus vaginalis* or *Corynebacterium vaginale*. Virulence factors of *G. vaginalis* include pili, production of a hemolytic cytotoxin and phospholipase A2 activity. It grows on blood or chocolate agar aerobically under 5% CO_2. Minute colonies appear in 24–48 hours and are hemolytic on human or rabbit blood agar. It is catalase-, oxidase-, indole- and urease-negative.

G. vaginalis is considered responsible for bacterial vaginosis, a mild but common condition characterised by raised vaginal pH > 4.5, foul-smelling discharge and the presence of '**clue cells'**, which are vaginal epithelial cells with their surface studded with numerous small bacteria (Fig. 39.8). Bacterial vaginosis is also associated with anaerobic bacteria, particularly *Mobiluncus* and the diagnosis is made on microscope criteria of a Gram smear of a high vaginal swab using the **Nugent score**. Metronidazole is effective in treatment.

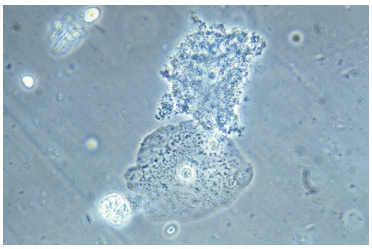


Fig. 39.8 Photomicrograph of clue cells from a vaginal smear; these are epithelial cells that have bacteria adherent to their surface, obscuring their borders, and imparting a stippled appearance (*Source:* Public Health Image Library, ID 14574 / M. Rein / CDC)

MORAXELLA (BRANHAMELLA) CATARRHALIS

Gram-negative cocci, usually arranged in pairs, growing readily on nutrient agar at 18–42°C, producing non-pigmented colonies and not fermenting sugars were formerly known as *Neisseria catarrhalis.* They have been reclassified as *Branhamella*, and again as *Moraxella catarrhalis*. They are a part of the normal pharyngeal flora but can cause respiratory infections, including otitis media, sinusitis, tracheobronchitis and pneumonia. As many strains produce beta-lactamases, penicillins are not useful in treatment unless given in combination with clavulanate or sulbactam.

It is the third most common cause of otitis media (after *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae*). It is a more common cause of pulmonary disease in COPD and in the elderly than previously thought. For diagnosis, sputum from lung disease is used for culture in 5% CO_2, which yields typical colonies on modified Thayer–Martin agar; gram-negative diplococci can be detected by direct microscopy. The bacteria can also be detected by PCR and by a commercial test kit. *Moraxella* infection is treated with antibiotics that do not induce the production of beta-lactamases (amoxicillin-clavulanate).

MORAXELLA LACUNATA

Also known as the Morax–Axenfeld bacillus, these are short, plump, gram-negative bacilli usually arranged in pairs. They are non-flagellated but have been reported to be sluggishly motile. Strictly aerobic, they grow on ordinary media. They are oxidase- and catalase-positive, indole- and H_2S-negative, and non-fermentative. They cause angular conjunctivitis.

LISTERIA MONOCYTOGENES

Listeria monocytogenes is a short, non-sporing, gram-positive bacillus. It has a **slow, tumbling motility when grown at 25°C** but is non-motile at 37°C. This is because peritrichous flagella are produced optimally at 20–30°C but not at 37°C. It is aerobic or microaerophilic. It grows in 5–10% CO_2 between 30°C and 37°C but slow growth occurs even at 4°C. Colonies are hemolytic on blood agar. *L. monocytogenes* ferments glucose, maltose, L-rhamnose and alpha-methyl D-mannoside, producing acid without gas. It is catalase-positive. It grows in the presence of 0.1% potassium tellurite, 10% salt and at pH 9.6.

Epidemiology

L. monocytogenes is widely distributed in nature. It has been isolated from mammals, birds, fish, ticks and crustacea. It occurs as a saprophyte in soil, water and sewage. Most human infections are caused by **serovars 1/2a or 1/2b and 4b**. Human infection occurs by (i) contact with infected animals, (ii) inhalation of contaminated dust or (iii) ingestion of contaminated milk or food. **Outbreaks of food-borne listeriosis** occurs due to its ability to grow at refrigeration temperature, low pH and high salt concentration (adopted for food preservation).

Monocytosis is a predominant feature of human listeriosis. Instillation into the eyes of rabbits produces keratoconjunctivitis (**Anton test**).

Pathogenicity

Listeriosis in human beings may present as follows:

- **Abortion** or **stillbirth** in pregnant women.
- **Meningitis or sepsis in neonates** due to perinatal transmission, which can be of early or late onset. Infants acquire infection in utero or by inoculation through an infected birth canal.
- **Meningoencephalitis** in the immunocompromised and the elderly by ingesting contaminated food.

The bacteria are invasive and produce an important virulence factor, **listeriolysin O,** which allows the bacterium to escape from cell membranes and contributes to septicemia. These organisms can cross the blood–brain barrier to cause meningitis and encephalitis. Cell-mediated immunity involving macrophages activated by T lymphocytes confers protection.

Asymptomatic infection of the female genital tract may cause infertility. Listeriosis may also present as abscess, conjunctivitis, pharyngitis, urethritis, pneumonia, infectious mononucleosis-like syndrome or endocarditis.

Laboratory Diagnosis

Appropriate specimens from various infections may be examined for diagnosis. Cervical and vaginal secretions, lochia, meconium, cord blood,

blood and cerebrospinal fluid may be cultured on blood agar and incubated in 5% CO_2 environment. Identification is by biochemical tests as shown in Table 39.1. **Cold enrichment** at 4°C in tryptose phosphate or thioglycolate broth and subcultures at weekly intervals for 1–6 months increases the rate of isolation. Antibody to listeriolysin O aids diagnosis.

Table 39.1 Differential features of non-sporing gram-positive bacilli

	L. monocytogenes	*E.rhusiopathiae*	Diphtheroids	Lactobacilli
Beta-hemolysis	+	–	±	–
Catalase	+	–	±	–
Motility	+ (at 25°C)	–	–	–
H_2S production	–	+	–	–

Treatment

Ampicillin, cotrimoxazole and gentamicin are effective. **Cephalosporins are not recommended**. Meningitis is treated with ampicillin.

Prevention

Proper food handling and personal hygiene are important. The condition needs to be recognised early in pregnancy.

ERYSIPELOTHRIX RHUSIOPATHIAE

Erysipelothrix rhusiopathiae is a slender, non-motile, non-sporing, non-capsulated, gram-positive bacillus with a tendency to form long filaments. It is microaerophilic on primary isolation but on subculture, grows as an aerobe or facultative anaerobe. It grows on ordinary media and is catalase-negative. Black colonies develop in tellurite media.

E. rhusiopathiae is a natural parasite of many animals. In humans, it causes **erysipeloid**. Human infection usually occurs on the hands or fingers due to direct inoculation at the site of a cut or abrasion in persons handling animals, fish or animal products. The lesions are painful, edematous and erythematous, usually involving the local lymph nodes and joints. **Occasional cases of endocarditis have been reported**. The bacillus is sensitive to penicillin, erythromycin and broad-spectrum antibiotics. It is

intrinsically resistant to vancomycin.

Key points

- *Yersinia pestis* is the causative agent of plague, which is a zoonotic disease. *Y. pestis* is naturally parasitic in rodents. Infection is transmitted to humans through the bite of an infected flea. Three clinical variants of plague are bubonic, septicemic and pneumonic plague. It is a fatal infection which can occur in epidemic forms.
- *Alcaligenes* are aerobic, oxidase-positive, catalase-positive rods exhibiting motility and not producing acid from carbohydrates in conventional culture media.
- *Chromobacterium* are gram-negative bacilli oxidase- and catalase-positive.
- *Flavobacterium* spp. are gram-negative bacilli, catalase-positive, oxidase-positive, and grow on ordinary culture media producing a yellow pigment. *F. meningosepticum* occasionally causes meningitis in infants.
- *Klebsiella granulomatis* is the causative organism of granuloma inguinale (donovanosis). The coccoid, rod-shaped organisms are gram-negative.
- The *Acinetobacter* species are gram-negative coccobacilli that are non-motile and strict aerobes. They are an important cause of nosocomial infections and are multidrug-resistant.
- Rat bite fever is caused by two different bacilli: *Streptobacillus moniliformis* and *Spirillum minus*.
- Members of *Campylobacter* are curved, gram-negative, oxidase-positive, motile, rod-shaped microaerophilic organisms. *Campylobacter jejuni* is a common cause of enteritis in humans and animals.
- *Helicobacter pylori* causes duodenal and stomach ulcers and is associated with gastric adenocarcinoma and lymphoma. These organisms produce urease.
- Members of the genus *Legionella* are catalase-positive, weakly oxidase-positive, motile, gram-negative bacilli whose natural habitat is water.
- *Legionella pneumophila* causes Legionnaires' disease and Pontiac fever. Infection is acquired by aerosol droplets.
- *Gardnerella vaginalis* is a rod-shaped bacterium which is non-motile, oxidase-negative, catalase-negative and gram-negative. It is considered to be a cause of vaginosis.
- Members of the genus *Moraxella* are rod-shaped bacteria, which are non-motile, obligate aerobes, both catalase- and oxidase-positive, asaccharolytic and gram-negative.
- *Erysipelothrix rhusiopathiae* is a rod-shaped bacterium which is non-motile, non-sporing, microaerophilic and gram-positive.
- *Listeria monocytogenes* is a gram-positive coccobacillus found in unpasteurised milk and many raw foods. It causes food-borne gastroenteritis, bacteremia in pregnant women and the immunocompromised and early- and late-onset infections in infants.

Questions

Short Answers

1. *Listeria monocytogenes*
2. Donovanosis
3. *Helicobacter pylori*
4. Rat bite fever
5. *Legionella pneumophilia*
6. *Acinetobacter* species
7. *Gardnerella vaginalis*
8. *Campylobacter* species

Part IV: Virology

Part IV: Virology

Chapter 40

Introduction to Virology

Chapter navigation

- Viral structure
- Viral genetics
- Classification of viruses

INTRODUCTION

Viruses do not fall strictly into the category of unicellular microorganisms as they do not possess cellular organisation. They are the smallest living organisms barring prions and are composed of only one nucleic acid, which is either a DNA or an RNA. They are ultramicroscopic structures with sizes ranging from 20 nm to 300 nm. Some of their defining characters are:

- They do not have cellular organisation.
- They contain only one type of nucleic acid, either DNA or RNA, but never both.
- They are obligate intracellular parasites.
- They lack the enzymes necessary for protein and nucleic acid synthesis and are dependent for replication on the synthetic machinery of host cells.
- They multiply by a complex process and not by binary fission.
- They are generally unaffected by antibacterial antibiotics.

The main differences between viruses and other microorganisms are presented in Table 40.1.

Table 40.1 Properties of prokaryotes and viruses

	Organised cell	Growth in artificial media	Binary fission	DNA and RNA	Ribo-somes	Sensitivity to antibiotics	Sensitivity to interferons
Viruses	–	–	–	–	–	–	+
Bacteria	+	+	+	+	+	+	_
Mycoplasmas	+	+	+	+	+	+	_
Rickettsiae	+	+	+	++	+	+	_
Chlamydiae	+	–	+	+	+	+	+

Viruses are responsible for a large number of human diseases, ranging from the common cold to the highly fatal rabies or AIDS. They may be sporadic like mumps, endemic like infectious hepatitis, epidemic like dengue fever or pandemic like influenza and the recent coronavirus. They may be geographically localised (arbovirus diseases) or may occur worldwide (like influenza). They have been associated with cancers (HPV) and other metabolic syndromes (e.g., diabetes with Coxsackievirus B).

MORPHOLOGY

Size

The extracellular infectious virus particle is called the **virion**. Viruses are much smaller than bacteria—too small to be seen under the light microscope (hence called ultramicroscopic) and only **seen under the electron microscope**. Some of the larger viruses, such as poxviruses, can be seen under the light microscope when suitably stained (Fig. 40.1).

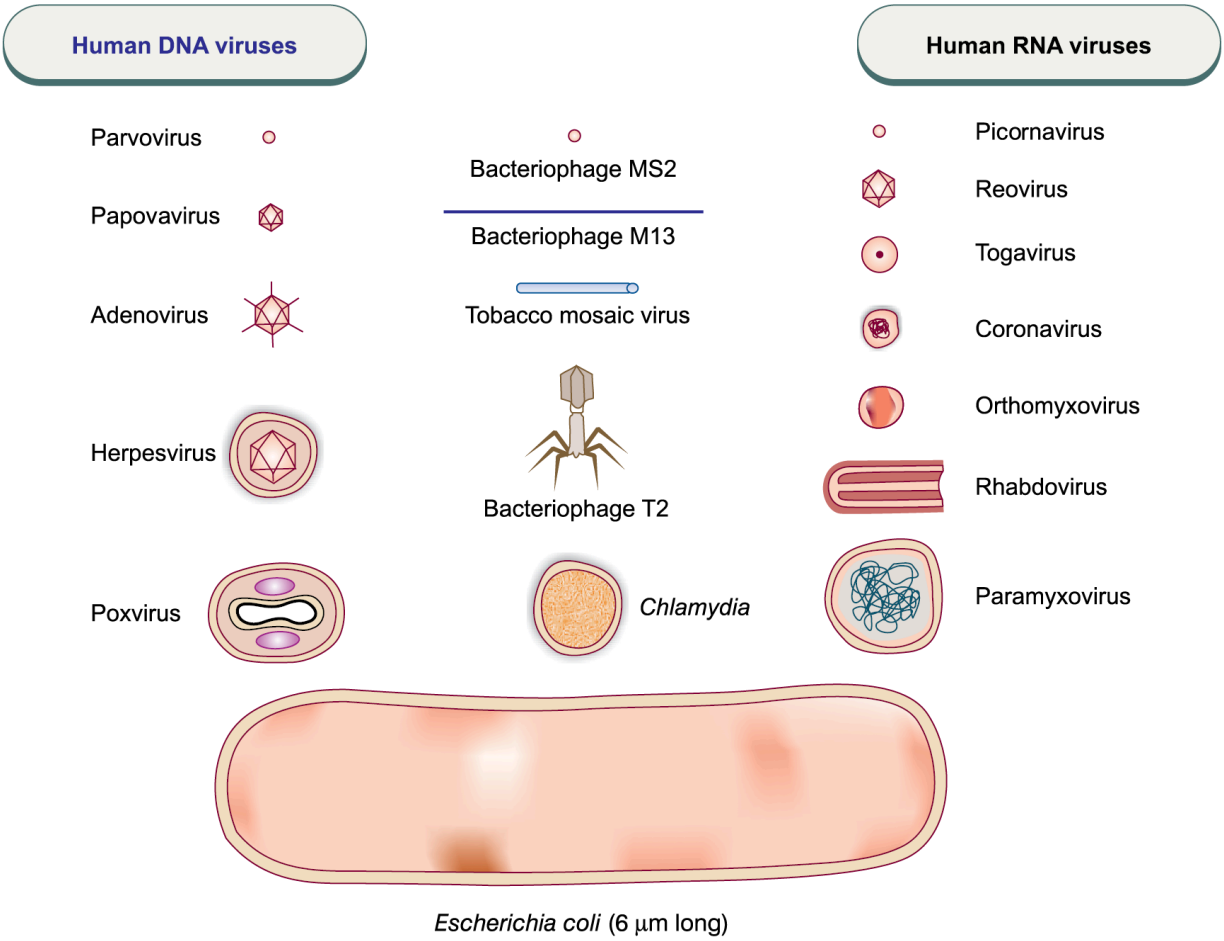


Fig. 40.1 Relative sizes of representative viruses, bacteriophages (bacterial viruses) and bacteria, including chlamydia

Viruses vary widely in size. The largest (poxvirus) measures about 300 nm and is as large as the smallest bacteria (mycoplasma). The smallest viruses (e.g., parvovirus) measures about 20 nm. Electron microscopy is used to estimate the size of virus particles.

Structure

Capsid

The virion consists essentially of a nucleic acid surrounded by a protein coat, the capsid. The capsid with the enclosed nucleic acid is known as the **nucleocapsid**.

The capsid is composed of a large number of **capsomers** which form its morphological units. Capsids are polypeptide molecules arranged symmetrically to form an impenetrable shell around the nucleic acid core.

One of the main functions of the capsid is to introduce the viral genome into host cells by adsorbing readily to cell surfaces.

Three kinds of **symmetry** are seen in the capsid:

i) **Icosahedral (cubical)** An icosahedron is a polygon with **12 vertices or corners and 20 facets or sides**. Each facet is in the shape of an equilateral triangle. Two types of capsomers constitute the icosahedral capsid—the pentagonal capsomers at the vertices **(pentons**) and the hexagonal capsomers making up the facets (hexons). There are always 12 pentons but the number of **hexons v**aries with the virus group.

ii) **Helical** In **nucleocapsids with helical symmetry**, the capsomers and nucleic acid are wound together to form a helical or spiral tube. In some, like the tobacco mosaic virus, the tube may be rigid; in animal viruses, the tubular nucleocapsid is pliable and may be coiled on itself. Not all viruses show the typical icosahedral or helical symmetry.

iii) **Complex** Some, like the poxviruses, exhibit complex symmetry.

Envelope

Virions may be enveloped or non-enveloped (naked). The envelope or outer covering is derived from the host cell membrane when the progeny virus is released by budding. The envelope is made of lipoprotein (Fig. 40.2). The lipid is largely of host cell origin while the protein is virus-coded. Protein subunits may be seen as projecting spikes on the surface of the envelope. These structures are called **peplomers** (from *peplos*, meaning 'envelope'). A virus may have more than one type of peplomer. The influenza virus carries two kinds of peplomers: the **hemagglutinin,** which is a triangular spike and the **neuraminidase,** which is a mushroom-shaped structure.

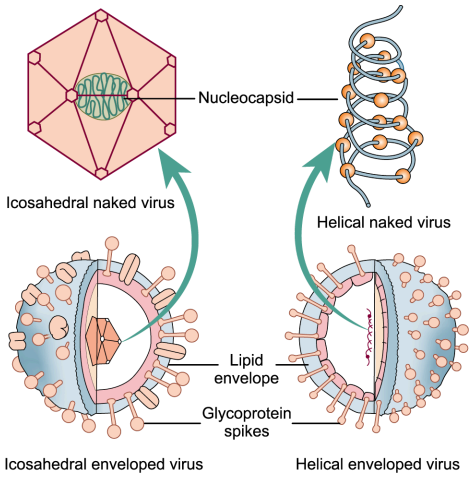


Fig. 40.2 Illustration of viral particles

Envelopes confer chemical, antigenic and biological properties on viruses. Enveloped viruses are susceptible to the action of lipid solvents like ether, chloroform and bile salts. Specific neutralisation of virus infectivity depends on antibodies to the surface antigens. Biological properties such as attachment to host cell surface or hemagglutination depend on the envelope.

Shape

This varies among different groups of viruses. Viruses are roughly **spherical**. Some are **irregular** and **pleomorphic**. The rabies virus is **bullet-shaped**, ebola virus and hepatitis viruses, **filamentous** (Fig. 40.3) and poxviruses, **brick-shaped**; the tobacco mosaic virus is **rod-shaped**.

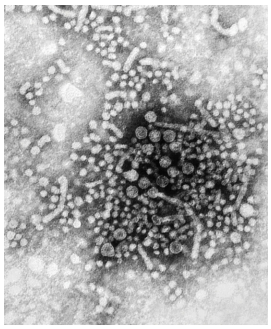


Fig. 40.3 Negative-stained transmission electron microscopic (TEM) image shows the presence of numerous filamentous forms of hepatitis B (HBV) virions (*Source:* Public Health Image Library, ID 10229 / Betty Partin / CDC)

Viral Components

Nucleic acid

Viruses contain only one type of nucleic acid, either single or double-stranded DNA or RNA. In this respect, viruses are unique, for nowhere else in nature is genetic information solely carried by RNA. Viral nucleic acids may be extracted by treatment with detergents or phenol. An extracted picornavirus and papovavirus nucleic acid is infectious to host cells.

Proteins

Viruses also contain proteins which make up the capsid. Viral protein, besides protecting the nucleic acid, also determines the antigenic specificity of the virus.

Lipids

Enveloped viruses contain lipids derived from the host cell membrane.

Others

Some viruses also contain small amounts of carbohydrate. Most viruses do not possess any enzymes for the synthesis of viral components or for energy production; some have other **enzymes,** for example, neuraminidase in the influenza virus. Retroviruses have a unique enzyme, RNA-dependent DNA polymerase or 'transcriptase' which can transcribe RNA into DNA.

RESISTANCE OF VIRUSES TO PHYSICAL AND CHEMICAL AGENTS

Viruses are resistant to the following:

- ***Temperature*** With few exceptions, viruses are very heat-labile. There are individual variations but in general, they are inactivated within seconds at 56°C, within minutes at 37°C and within days at 4°C. They are stable at low temperatures. For long-term storage, they are kept frozen at –70°C. The method for prolonged storage is lyophilisation or freeze-drying (drying the frozen virus under vacuum). Lyophilised viruses can be stored for years and reconstituted when required by adding a suitable virus medium. Some viruses (such as the poliovirus) do not withstand freeze-drying.

- ***pH*** Viruses vary greatly in their resistance to acidity. For example, enteroviruses are very resistant to acid pH while rhinoviruses are very susceptible. All viruses are disrupted under alkaline conditions.
- ***Radiations*** Viruses are inactivated by sunlight, UV rays and ionising radiation.
- ***Disinfectants*** Viruses are, in general, more resistant than bacteria to chemical disinfectants, probably because they lack enzymes. Phenolic disinfectants are only weakly virucidal. 50% glycerol saline acts as a preservative for many viruses (e.g., vaccinia and rabies). Molar concentrations of certain salts ($MgCl_2$ and Na_2SO_4) also protect some viruses (e.g., poliovirus) against heat inactivation. **The most active antiviral disinfectants are oxidising agents such as hydrogen peroxide, potassium permanganate and hypochlorites.** Organic iodine compounds are actively virucidal. Chlorination of drinking water kills most viruses but its efficacy is greatly influenced by the presence of organic matter. Some viruses such as **hepatitis virus** and **polioviruses are relatively resistant to chlorination**. Formaldehyde and beta-propiolactone are actively virucidal and are commonly employed for the preparation of killed viral vaccines. Non-enveloped viruses are more resistant to disinfectants.
- ***Lipid solvents*** The action of lipid solvents such as ether, chloroform and bile salts is selective, the enveloped viruses being sensitive and the naked viruses resistant to them. This selective action is useful in the identification and classification of viruses.
- ***Antibiotics*** These are absolutely ineffective against viruses, a property used to eliminate bacteria from the specimen before virus isolation.

VIRAL REPLICATION

The genetic information necessary for viral replication is contained in the viral nucleic acid. However, viruses lack biosynthetic enzymes and therefore, depend on the synthetic machinery of the host cell for replication.

The viral replication cycle can be divided into six sequential phases (Fig. 40.4), though the phases may sometimes overlap.

The early events that occur in the virus replicative cycle are:

Attachment → Penetration → Uncoating

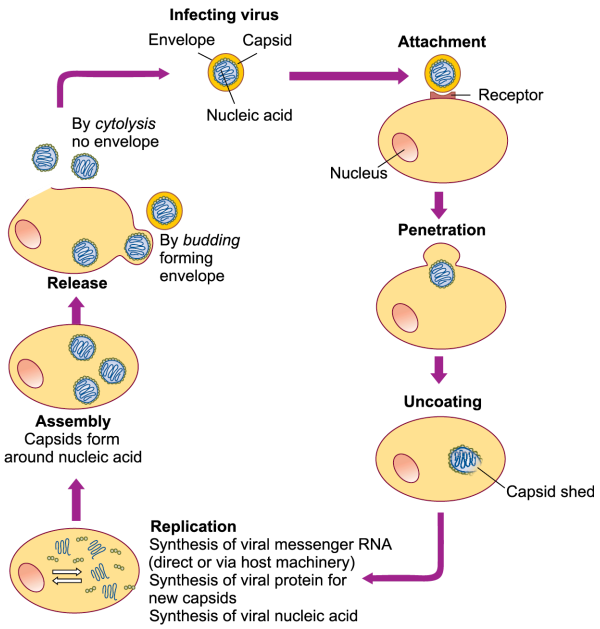


Fig. 40.4 Stages in the infection of a host's cell and replication of a virus

1. Attachment and Adsorption

Virions come into contact with cells by random collision but adsorption takes place only if there is an affinity between the two. The cell surface should contain specific receptor sites to which the virus can gain attachment. This **determines the host range**. Some examples are given below:

- Influenza virus—the hemagglutinin on the virus surface gets attached to glycoprotein receptor sites on the surface of the respiratory epithelium. Destruction of the receptor sites by RDE prevents viral adsorption.
- HIV—attachment is between the CD4 receptor on host cells and the viral surface glycoprotein gp120.
- Polioviruses—the receptor is the lipoprotein present on the surface of primate cells.

Differences in susceptibility to viral infection are, to a large extent, based on the presence or absence of receptors on cells. If the phase of adsorption can be bypassed, cells normally insusceptible to viruses may be rendered susceptible to them.

2. Penetration

Animal cells do not have rigid cell walls so the whole virus can enter into them.

- Virus particles may be engulfed by a process known as pinocytosis.
- Alternatively, in enveloped viruses, the viral envelope may fuse with the plasma membrane of the host cell and release the nucleocapsid into the cytoplasm.

3. Uncoating

This is the process of stripping the virus of its outer layers and capsid so that the nucleic acid is released into the cell. With most viruses, uncoating is effected by the action of lysosomal enzymes of the host cell.

The next phase of viral replication is gene expression and genome replication.

4. Synthesis of DNA/RNA

This phase includes the synthesis not merely of the viral nucleic acid and capsid protein, but also of enzymes necessary for the various stages of viral synthesis, assembly and release. In addition, certain **'regulator proteins'** are also synthesised, which serve to shut down normal cellular metabolism and direct the sequential production of viral components. The site of viral synthesis depends on the type of virus.

- **DNA viruses synthesise their nucleic acid in the host cell nucleus**. The exceptions are the poxviruses, which synthesise all their components in the host cell cytoplasm.
- **RNA viruses synthesise all their components in the cytoplasm**. Exceptions are orthomyxoviruses, some paramyxoviruses and

retroviruses whose components are synthesised partly in the nucleus. Viral protein is synthesised only in the cytoplasm.

Steps in biosynthesis

i) **Early transcription** of messenger RNA (mRNA) from the viral nucleic acid.

ii) **Early translation** of the mRNA into 'early proteins'. These 'early or non-structural proteins' are enzymes that initiate and maintain the synthesis of virus components. They may also induce shutdown of host protein and nucleic acid synthesis.

iii) **Replication** of viral nucleic acid (following DNA synthesis and late transcription).

iv) **Late synthesis** of 'late' or structural proteins, which are the components of daughter virion capsids.

The critical step in viral biosynthesis is the transcription of mRNA from the viral nucleic acid. Once this is achieved, the host cell resources can be utilised for translating mRNA into viral components. Depending on the structure of their genome, viruses use different strategies for the transcription of mRNA.

Replication mechanisms

Viruses have been categorised into six classes by Baltimore (1970) based on their replication mechanisms.

Class 1 In the case of fully double-stranded DNA viruses (such as **adeno, herpes and papovaviruses**), the DNA enters the host cell nucleus and uses the host cell enzymes for transcription. The extracted DNA from these viruses is infectious. With hepadnaviruses, which have a partially double-stranded DNA, the duplex is completed by a viral DNA polymerase inside the host cytoplasm. The mature DNA then moves into the nucleus, to be transcribed by host transcriptases. Extracted hepadnavirus DNA is not infectious. Poxviruses, which replicate in the cytoplasm, form mRNA using polymerases contained in the virion itself. Poxvirus DNA is not infectious.

Class 2 In single-stranded DNA viruses (e.g., **parvovirus**), the DNA molecule moves into the host cell nucleus and is converted into the duplex form. Transcription is achieved by host enzymes.

Class 3 In reoviruses, the double-stranded RNA is transcribed to mRNA by viral polymerases.

Class 4 Depending on the method of mRNA transcription, single-stranded RNA viruses are classified into two categories. In **the positive strand (plus strand, positive sense**) RNA viruses, the viral RNA itself act as the mRNA. **Viral RNA is infectious** by itself and is translated directly into viral proteins in the host cell cytoplasm (e.g., picorna and togaviruses).

Class 5 In the **negative strand (minus sense) RNA viruses** (e.g., rhabdo, orthomyxo and paramyxoviridae), the RNA is 'antisense', with polarity opposite to mRNA. They possess their own RNA polymerases for mRNA transcription. Extracted nucleic acids from these viruses are **not infectious**.

Class 6 Retroviridae exhibit a unique replicative strategy. Their single-stranded RNA genome is converted into an RNA:DNA hybrid by the viral **reverse transcriptase** (RNA-directed DNA polymerase) enzyme. Double-stranded DNA is then synthesised from the RNA:DNA hybrid. The double-stranded DNA form of the virus (provirus DNA) is integrated into the host cell chromosome. This integration may lead to the transformation of the cell and the development of neoplasia.

5. Maturation

Assembly of progeny virions follows the synthesis of viral nucleic acid and proteins. Virion assembly may take place in the host cell nucleus or cytoplasm. Herpes and adenoviruses are assembled in the nucleus, while picorna and poxviruses are assembled in the cytoplasm. At this stage, the non-enveloped viruses are present intracellularly as fully developed virions, but in the case of enveloped viruses, only the nucleocapsid is complete. **Viral envelopes are derived from the host cell membrane** during the process of budding. Myxoviruses bud from the cell surface and their envelope is formed by the modified cytoplasmic membrane of the host cell. The incorporation of viral antigen (hemagglutinin) on the cell membrane endows the cell with the property of hemadsorption.

6. Release

Virus release may occur without cell lysis. Myxoviruses are released by a process of budding from the cell membrane over a period of time. The host

cell is unaffected and may even divide, with the daughter cells continuing to release virions. In some viruses (e.g., varicella), transmission occurs directly from cell to cell, very little free virus being demonstrable extracellularly in the medium. However, several viruses cause host cell lysis at the time of release, e.g., non-enveloped viruses and bacteriophages.

Eclipse phase From the stage of penetration up to the appearance of mature progeny virions, the virus cannot be demonstrated inside the host cell. This period, during which the virus seems to disappear, is known as the 'eclipse phase'. The time taken for a single cycle of replication is about 15–30 minutes for bacteriophages and about 15–30 hours for animal viruses. A single infected cell may release a large number of progeny virions. This is seen readily in bacteriophages (the number of phages produced per infected bacterium is called **burst size**); it is difficult to assess in animal viruses that are released over a prolonged period.

Abnormal Replicative Cycles

- A proportion of daughter virions produced may not be infective. This is due to defective assembly. Such **'incomplete viruses'** are seen in large proportions when cells are infected with a high dose of the influenza virus. The virus yield will have a high hemagglutinin titre but low infectivity. This is known as the **von Magnus phenomenon**.
- Virus infection in some cells does not lead to the production of infectious progeny. In such cells **(non-permissive cells)**, the viral components may be synthesised but maturation or assembly is defective. As a result, either no release occurs or the progeny is non-infectious. This is known as **abortive infection**. Here, the defect is in the type of cell and not in the parental viruses.
- Some viruses are **genetically defective** in that when they infect cells, they are unable to give rise to fully formed progeny. Yield of progeny virions occurs only if the cells are simultaneously infected with a helper virus, which can supplement the genetic deficiency. For example, some strains of the Rous sarcoma virus (RSV) cannot code for the synthesis of the viral envelope. When

RSV infects a cell that harbours a helper virus (e.g., avian leukosis virus), infectious progeny result, the helper virus contributing to the synthesis of the envelope. The envelope antigen of progeny RSV will, therefore, be determined by the type of helper virus. Other examples of defective viruses are the **hepatitis D virus** and **adeno-associated satellite viruses (AAV)** which replicate only in the presence of their helper viruses, hepatitis B and adenoviruses respectively. Viruses that are genetically deficient and, therefore, incapable of producing infectious virions without the helper activity of another virus are known as '**defective viruses**'.

VIRAL GENETICS

Several properties of viruses, such as virulence and antigenicity, are under genetic control.

The two main mechanisms of genetic modification in viruses are mutation and recombination. In addition, viruses may exhibit many non-heritable variations due to gene product interactions.

Mutation

The frequency of mutation in viruses is about 10^{-4} to 10^{-8}, approximately the same as in bacteria. Mutations, therefore, occur during every viral infection. Mutation may occur spontaneously or may be induced by mutagens, physical agents such as irradiation or chemical agents such as 5-fluorouracil.

Types of mutants

Some mutations of clinical and laboratory interest are those affecting virulence, host range, antigenicity and pock or plaque morphology.

Conditional lethal mutant This class of mutants is of great importance in laboratory studies. These mutants grow only under certain favourable conditions (called **permissive conditions**) and cannot grow under certain other conditions (called **non-permissive or restrictive conditions**), which may be lethal to these viruses. There are different types of conditional lethal mutants.

Ts mutant Ts or **temperature-sensitive mutant** is most widely employed in genetic studies. These can grow at a low (permissive) temperature (28–31°C) but not at a high (restrictive) temperature (37°C). The advantage here is that by using a single selective test (temperature sensitivity), a large number of mutants with mutations in different genes may be obtained.

Host-dependent mutants These can grow in permissive cells but cause abortive infection in non-permissive cells.

Genetic Interactions

Recombination

Genetic recombination may occur when two different but related, viruses infect a cell simultaneously. The two viruses exchange segments of nucleic acids so that a hybrid results, possessing genes from both parents. Such recombinants breed true thereafter. Recombinants may occur between:

- two active (infectious) viruses;
- one active and one inactive virus; or
- two inactive viruses.

Reassortment When two different strains of the same virus (such as vaccinia or influenza) possessing distinctive markers (such as pock morphology or antigenic properties) are grown together, recombinants or **reassortant viruses** which possess the distinctive properties of both parents may be derived.

Reactivation

When a cell is 'infected' with an active virus and a different but related inactivated virus, progeny possessing one or more genetic traits of the inactivated virus may be produced. This phenomenon is called **cross-reactivation** or **marker rescue.** New antigenic variants of the influenza virus causing epidemics often do not grow well in eggs as compared to established laboratory strains. When such an epidemic strain (e.g., strain A_2) is grown in eggs along with a standard strain (e.g., strain A_1) inactivated by UV irradiation, a progeny may be obtained which has the antigenic characters of A_2 but the growth characteristics of A_1. **This finds application**

in the manufacture of the influenza virus vaccines.

When a cell is 'infected' with a large dose [high multiplicity of infection (high MOI)] of a single virus inactivated by UV irradiation, a live virus may be produced. This phenomenon is called **multiplicity reactivation**. There is the potential danger of multiplicity reactivation taking place following the administration of UV irradiated vaccines. **UV irradiation is, therefore, not acceptable as a method of producing inactivated virus vaccines**.

As a general rule, virus capsids enclose viral nucleic acids. Sometimes, segments of host nucleic acid become encapsidated instead. For example, in a papovavirus capsid, a linear piece of host DNA roughly the same size as the papovavirus genome may be found. This is known as a **pseudovirion**.

Viral particles containing host DNA sequences are important because of their potential ability to transduce host genes from one cell to another. This could be exploited for correcting inborn errors of metabolism.

Non-genetic Interactions

Phenotypic mixing

When two different viruses multiply in a cell, some 'mix-up' may take place during assembly, whereby the genome of one virus may be surrounded by a capsid belonging partly or entirely to the other virus. This is known as **transcapsidation** or phenotypic mixing. When phenotypic mixing occurs between two enveloped viruses, resulting in the sharing of peplomers, **mosaic envelopes result**.

Genotypic mixing

This heterozygosis results from the incorporation of more than one complete genome into a single virus particle. There is no recombination between the different genomes and thus two kinds of viral progeny are formed on passage.

Complementation

This is a functional interaction between the gene products (proteins specified by genes) of two viruses, one or both of which may be defective, resulting in the multiplication of one or both under conditions in which replication would not ordinarily occur.

Interference

The usual result of mixed or multiple infections of cells is interference, in which infection of a cell by one virus inhibits the simultaneous or subsequent infection by another. The most important **mediator of interference is the interferon**, a soluble cellular product. Interference is seen with myxoviruses and enteroviruses for which cell receptors are important for the initiation of infection. Another type of interference is 'autointerference', in which a high multiplicity of infection inhibits the production of infectious progeny.

Viral interference has been applied in the field in controlling poliomyelitis outbreaks by introducing into the population, the live attenuated poliovirus vaccine. The vaccine virus interferes with the spread of wild poliovirus and halts the outbreak. On the other hand, interference by pre-existing enteric viruses may pose a problem in live poliovirus vaccination.

CLASSIFICATION AND NOMENCLATURE OF MEDICALLY IMPORTANT VIRUSES

Viruses are classified into two main divisions depending on the type of nucleic acid they possess:

1. **Riboviruses** are those containing RNA.
2. **Deoxyriboviruses** are those containing DNA.

Further classification is based on other properties such as the **strandedness** of nucleic acid, symmetry of the **nucleocapsid**, presence of an envelope, size and shape of the virion and the number of capsomers. Short descriptions of the major groups of viruses are given in Table 40.2.

Table 40.2 Classification of viruses

Classification of viruses				
	Family	**DNA structure**	**Envelope**	**Members causing human disease**
DNA Viruses				
1.	Poxviridae	ds, linear	±	Variola virus, vaccinia virus, cowpox
				Molluscum contagiosum monkeypox
2.	Herpesviridae	ds, linear	Yes	Herpes simplex 1 and 2, EBV, CMV, varicella zoster, HSV-6,7,8
3.	Adenovirus	ds, linear	No	Human adenoviruses A-F

4.	Papillomas	circular	No	Human papillomavirus
5.	Polyoma	ds, circular	No	JC virus, BK virus
6.	Hepadnaviridae	ds, incomplete circular	Yes	Hepatitis B virus
7.	Parvoviridae	ss, linear	No	B19 virus
RNA Viruses				
1.	Picornaviridae	ss, linear	No	Enteroviruses—poliovirus, coxsackie, echovirus
2.	Orthomyxoviridae	ss, linear	Yes	Influenza virus A–C
3.	Paramyxoviridae	ss, linear	Yes	Paramyxoviruses—mumps and parainfluenza viruses Morbillivirus—measles virus, pneumoviruses—RSV
4.	Togaviridae	ss, linear	Yes	Alpha (group A)—Chikungunya virus Rubiviruses—rubella virus
5.	Flaviviridae	ss, linear	Yes	Flaviviruses—yellow fever, dengue viruses Japanese encephalitis virus, hepatitis C virus
6.	Bunyaviridae	ss, circular	Yes	Sandfly fever, hantaviruses
7.	Arenaviruses	ss, circular	Yes	LCM, Lassa fever
8.	Rhabdoviridae	ss, linear	Yes	Vesiculovirus—Chandipura virus, Lyssavirus—rabies virus
9.	Reoviridae	ds, linear	No	Human rotavirus
10.	Coronaviridae	ss, linear	Yes	Human coronaviruses, SARS virus, MERS-CoV, COVID-19
11.	Retroviridae	ss, linear	Yes	HIV 1–2, HTLV1-2
12.	Calciviridae	ss, linear	No	Norwalk virus, hepatitis E virus
13.	Filoviridae	ss, linear	Yes	Marburg, Ebola viruses
14.	Astroviridae	ss, linear	No	Human astroviruses
15.	Deltaviruses	ss, circular	Yes	Hepatitis deltavirus

DNA Viruses

1. ***Poxviridae family*** These are large, brick-shaped or ovoid viruses (300 × 240 × 100 nm) with a complex structure comprising lipid-containing outer coat, one or two lateral bodies and a core carrying a single, linear molecule of double-stranded DNA. Multiplication and maturation take place in the cytoplasm. The family is divided into several genera.
2. ***Herpesviridae family*** These are medium-sized viruses containing linear, double-stranded DNA. The icosahedral nucleocapsid (100 nm) has 162 capsomers and is surrounded by a lipid-containing envelope. Multiplication takes place in the nucleus and maturation by budding, through the nuclear membrane. Only one genus, *Herpesvirus*, has been characterised, while several members of the family await classification.
3. ***Adenoviridae family*** These are medium-sized (70–90 nm), non-enveloped, icosahedral viruses with 252 capsomers. Members have been classified into two genera: *Mastadenovirus* (mammalian adenoviruses) and *Aviadenovirus* (adenoviruses of birds).
4. ***Papovaviridae family*** These are small (40–55 nm), non-enveloped, double-stranded DNA viruses with 72 capsomers. Two genera have been identified: *Papillomavirus* and *Polyomavirus.*
5. ***Parvoviridae family*** These are very small (18–26 nm), non-enveloped viruses with 31 capsomers. The genome consists of single-stranded DNA. Three genera have been described: *Parvovirus, Adenosatellovirus* and *Densovirus*.
6. ***Hepadnaviridae family*** This consists of the human hepatitis type B virus and related viruses of animals and birds. (The name comes from *hepa* referring to the liver and DNA for DNA core.) The virion is spherical, 42 nm in diameter, consisting of a 27 nm core surrounded by an envelope having virus-specific antigens.

RNA Viruses

1. ***Picornaviridae family*** These are small (20–30 nm), non-enveloped, icosahedral viruses with a single-stranded RNA genome. Three genera are of medical importance:

- *Enterovirus*, including polio, coxsackie, echo and several other related viruses
- *Rhinovirus,* including human, bovine and equine rhinoviruses
- *Hepatovirus,* which includes Hepatitis A virus

2. ***Orthomyxoviridae family*** These are medium-sized (80–120 nm), spherical or elongated, enveloped viruses carrying hemagglutinin and neuraminidase peplomers. The genome consists of a single-stranded RNA in several (eight) pieces. Only one genus— *Influenzavirus*—has been recognised. Influenza virus type C possesses several distinctive features and may have to be separated into a new genus.
3. ***Paramyxoviridae family*** These are pleomorphic virions (150 nm) with lipid envelopes that have surface projections. The genome is an unsegmented single-stranded linear RNA. Three genera have been recognised:

    - *Paramyxovirus*, which consists of the Newcastle disease virus, mumps virus and parainfluenza viruses of humans, other mammals and birds
    - *Morbillivirus*, containing measles, canine distemper, rinderpest and related viruses
    - *Pneumovirus*, containing respiratory syncytial virus of humans and related viruses

4. ***Togaviridae family*** These are spherical viruses, 40–70 nm in size, with a lipoprotein envelope and single-stranded RNA genome. Most members multiply in arthropods as well as in vertebrates. Three genera have been described:

    - *Alphavirus*, consisting of viruses formerly classified as Group A arboviruses
    - *Rubivirus*, consisting of the rubella virus
    - *Pestivirus*, consisting of the mucosal disease virus, hog cholera virus and related viruses

5. **Flaviviridae family** Flaviviruses, formerly grouped under togaviridae as Group B arboviruses, have been classified as a separate family because of differences in their molecular structure and replication strategy.
6. ***Bunyaviridae family*** **These are** spherical, enveloped virions, 90–100 nm in size. They are arthropod-borne viruses. Five genera are established: the large genus *Bunyavirus* containing about 150 species, and four other genera—*Hantavirus*, *Nairovirus*, *Phlebovirus*, *Ukuvirus*—and many unassigned viruses.
7. ***Arenaviridae family*** **These are** spherical or pleomorphic viruses, 50–300 nm in size, containing a number of electron-dense ribosome-like particles, which give it a sandy appearance (hence the name *arena*, meaning 'sand' in Latin). Members are generally rodent parasites that cause persistent infection in the natural host but are capable of infecting human beings rarely, leading to severe hemorrhagic illness. Only one genus, *Arenavirus*, has been recognised. Species include lymphocytic choriomeningitis virus, Lassa and members of the Tacaribe complex.
8. ***Rhabdoviridae family*** These are bullet-shaped viruses, 130–300 nm long and 70 nm wide, with a lipoprotein envelope carrying peplomers. Two genera have been recognised:

    - *Vesiculovirus*, containing vesicular stomatitis virus, Chandipura virus (isolated from humans in India) and related species
    - *Lyssavirus*, containing the rabies virus and related viruses such as Lagos bat, Mokola, Duvenhage and others

Other genera that have been suggested include rhabdoviruses of insects and plants.

9. ***Reoviridae family*** These are icosahedral, non-enveloped viruses, 60–80 nm in size with double-layered capsids. Their genome consists of double-stranded RNA in 10–12 pieces. Three genera have been recognised:

    - *Reovirus*, containing reoviruses from humans, other mammals and birds

- *Orbivirus*, containing several species of arboviruses such as blue tongue virus, African horse sickness virus and Colorado tick fever virus
- *Rotavirus*, including human rotaviruses, calf diarrhea virus and related agents

Other genera may have to be defined to include plant and insect viruses belonging to this family.

10. ***Coronaviridae family*** **These are** pleomorphic, enveloped viruses around 100 nm in size, with unique club-shaped peplomers projecting as a fringe from the surface, resembling the solar corona (hence the name). Only one genus, *Coronavirus*, has been recognised. Members include human coronaviruses causing upper respiratory disease, SARS avian infectious bronchitis virus, calf neonatal diarrhea coronavirus, murine hepatitis virus and related viruses.
11. ***Retroviridae (re = reverse, tr = transcriptase) family*** These are RNA tumour viruses and related agents. Virions are icosahedral, about 100 nm in size, with lipoprotein envelopes. The characteristic biochemical feature is the presence of RNA-dependent DNA polymerase (reverse transcriptase) within the virus. Three subfamilies are recognised:

    - Oncovirinae, the RNA tumour virus group
    - Spumivirinae, the foamy virus group (*spuma* = foam)
    - Lentivirinae (*lenti* = slow), visna and maedi viruses of sheep belonging to the slow virus group

12. ***Caliciviridae family*** These are naked spherical particles (35–39 nm) with 32 cup-shaped depressions arranged in symmetry.
13. ***Filoviridae family*** These are long, filamentous, enveloped viruses (80 nm diameter and up to 14,000 nm long) with a helical nucleocapsid and a ss RNA genome. This group contains the *Marburg* and *Ebola* viruses which cause human hemorrhagic fevers.

VIROIDS

The term ‘viroid’ was introduced by Diener (1971) to describe a new class of subviral agents characterised by the apparent absence of an extracellular dormant phase (virion) and by a genome much smaller than those of known viruses. The infective agent is a protein-free, low-molecular-weight RNA, which is resistant to heat and organic solvents but sensitive to nucleases. Viroids have been shown to cause some plant diseases. It is possible that the causative agents of some animal and human diseases may turn out to belong to the class of viroids.

PRION

An unconventional, virus-like agent has been named **prion** (1982). Prions are proteinaceous infectious particles. It has been suggested that they may also be responsible for some chronic neurological and degenerative diseases in humans.

The causative agents of scrapie, kuru and Creutzfeldt–Jakob disease have been shown to be small particles (MW 50,000 and probably 4–6 nm in diameter) without any detectable nucleic acid, which are resistant to heat (90°C for three minutes), UV rays and nucleases. They are sensitive to proteases. Diseases caused by prions are discussed in Chapter 51.

Key points

- Viruses are obligate intracellular parasites because they are dependent on the synthetic machinery of the host cell for replication. They are considered to be the smallest ‘living units not affected by antibacterial antibiotics’.
- The extracellular infectious virus particle is the virion. Viruses are much smaller than bacteria and can be visualised directly by electron microscopy.
- Viruses have either DNA or RNA and never both together.
- Virions may be enveloped or non-enveloped. The envelope helps in attachment to the host cell surface or to erythrocytes.
- Protein subunits (peplomers) may occur as projecting spikes on the envelope surface (e.g., hemagglutinin and neuraminidase of the influenza virus).
- The viral multiplication (replication) cycle consists of six sequential phases (overlaps may occur):
    - Adsorption (attachment)
    - Penetration into the cell
    - Uncoating

    - Biosynthesis
    - Maturation
    - Release of progeny
- The virus cannot be demonstrated within the host cell from the stage of penetration up to the stage of release—the 'eclipse phase'.
- There are two main mechanisms for genetic modification in viruses, namely, mutation (which occurs during every viral infection) and recombination (which occurs when two different but related viruses simultaneously infect a cell).
- Non-genetic interactions between viruses may occur when two different viruses simultaneously infect a single cell.
- DNA viruses comprise the following families: Poxviridae, Herpesviridae, Adenoviridae, Papovaviridae, Parvoviridae and Hepadnaviridae.
- RNA viruses comprise the following families: Picornaviridae, Orthomyxoviridae, Paramyxoviridae, Togaviridae, Flaviviridae, Bunyaviridae, Arenaviridae, Rhabdoviridae, Reoviridae, Coronaviridae, Retroviridae, Caliciviridae and Filoviridae.
- A viroid is a subviral agent that does not have an extracellular dormant (virion) phase and has a genome that is much smaller than those of known viruses.
- Prions are proteinaceous infectious particles that appear to lack nucleic acids.

Questions

Essays

1. Describe the replicative cycle of a DNA virus.
2. Classify RNA viruses and describe their replication.

Short Answers

1. General characteristics of viruses
2. Viral multiplication
3. Hemagglutination in viruses
4. List five RNA viruses and the diseases caused by them
5. List DNA viruses and the diseases caused by them
6. Prions

Part IV: Virology

CHAPTER 41

Basic Concepts of Viral Infections

Chapter navigation

- Pathogenesis of viral infections
- Host response to viral infections
- Laboratory diagnosis of viral diseases

INTRODUCTION

Viral infections result in a wide range of effects, starting from no apparent cellular damage to rapid cell destruction in the host, leading to pathogenic changes. Some viruses, like the poliovirus, cause **cell death** (**cytocidal effect**) or **lysis** (**cytolysis**). Others may cause **cellular proliferation** (molluscum contagiosum) or **malignant transformation** (oncogenic viruses). In some instances, the virus and host cells enter into peaceful co-existence, both replicating independently without any cellular injury, resulting in a chronic diffuse infection; this is known as **steady-state infection**.

How the virus interacts with host cells (whether it is cytolytic, steady-state, latent, and/or integrated infection) and the mode of spread of the virus (whether local, primary hematogenous or secondary hematogenous) are influenced by various defence mechanisms of the body.

Action of Viruses on Cells

- During virus replication, **early** or **non-structural viral proteins** often cause the shutdown of host protein and DNA synthesis.
- Large amounts of **viral macromolecules** that accumulate in the infected cell may distort the cellular architecture and exert a toxic effect.

- The **permeability of plasma membranes** may be altered, releasing lysosomal enzymes and leading to autolysis.
- Many viruses produce alterations in the **cytoplasmic membrane** of infected cells.
- Some (such as the respiratory syncytial virus) cause fusion of adjacent cell membranes, leading to polykaryocytosis or **syncytium** formation.
- Virus-coded antigens may appear on the surface of infected cells. These antigens may confer new properties on the cells. For example, viral **hemagglutinin** appears on the surface of cells infected with the influenza virus and causes adsorption of erythrocytes to the cell surface (hemadsorption). **Virus-coded antigens** also appear on the surface of cells transformed by oncogenic viruses.

Certain viruses such as those causing measles and mumps, adenoviruses, cytomegalovirus and varicella virus cause damage to the chromosomes of host cells. Chromatid gaps and breaks in chromosome 17 occur frequently in cultured cells infected with adenovirus types 12 and 31.

Inclusion bodies

The most characteristic histological feature of virus-infected cells is the presence of inclusion bodies. These are structures with a distinct size, shape, location and staining properties that can be demonstrated in virus-infected cells under the light microscope (Fig. 41.1). Inclusion bodies may be crystalline aggregates of virions or made up of virus antigens present at the site of virus synthesis. Some inclusions represent degenerative changes produced by viral infections, which confer altered staining properties on the cell.

They may be situated in the cytoplasm (as with poxviruses), nucleus (herpesviruses) or both (measles virus). They are generally acidophilic and can be seen as pink structures when stained by the Giemsa method or with eosin methylene blue. Some viruses (e.g., adenovirus) form basophilic inclusions.

Demonstration of inclusion bodies helps in the diagnosis of some viral infections (Table 41.1).

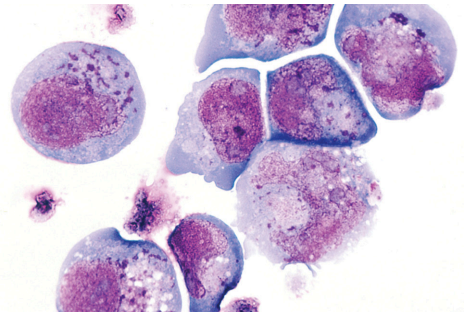


Fig. 41.1 Inclusion bodies in herpesvirus (Giemsa stain)

Table 41.1 Some viral inclusion bodies, their staining and sites in the cell

Name	Site	Nature	Disease
Negri bodies	Intracytoplasmic	Eosinophilic	Rabies
Guarnieri bodies	Intracytoplasmic	Acidophilic	Vaccinia
Molluscum bodies	Intracytoplasmic	Eosinophilic	Molluscum contagiosum
Cowdry type A inclusions	Intranuclear inclusion	Acidophilic	Herpesvirus, yellow fever virus
Type B inclusions	Intranuclear inclusion	Basophilic	Adenovirus, Poliovirus
Warthin-Finkeldey	Intracytoplasmic and intranuclear	Eosinophilic	Measles

PATHOGENESIS OF VIRAL INFECTIONS

Depending on the clinical outcome, viral infections can be classified as follows:

- **Subclinical** (inapparent) infections
- **Clinical** (apparent or overt) infections, which may be acute, subacute or chronic
- **Latent** infections, which remain quiescent for long periods and may be of the following types:
    - **Recurrent** Herpes simplex and herpes zoster are examples of latent infections in which clinical manifestations appear after prolonged periods of quiescence, during which, the viruses persist in the nerve root ganglia.
    - **Persistent** Tolerant infection occurs when the virus is readily demonstrable in the tissues of the host but neither disease nor immune response develops. The host is immunologically tolerant to the virus as a result of congenital or neonatal infection.
    - **Slow infection** Slowly progressive infection (incubation period is unusually long) is seen in neurological diseases such as **kuru** in human beings and scrapie in sheep.
    - Infections that are caused by **oncogenic** viruses.

- **Special type of latency** The **HIV** leads to a special type of latency with an initial asymptomatic period followed by progressive, immune damage-causing secondary diseases that end fatally after many years.
- **Abortive** Some infections may be **abortive,** leading to non-progression to clinical infection. *Abortive*replication and reactivation in vitro are seen in respiratory syncytial*virus*(RSV)*infection*in palivizumab-treated HeLa cells.

Routes of Entry

Viruses enter the body through the respiratory and alimentary tracts, skin, conjunctiva and the genital tract. Many viruses are transmitted vertically, i.e., from mother to child.

1. The **respiratory tract** offers the most important portal of entry for viruses. A large number of viruses can infect the cells of this tract. **Influenza** and **rhinoviruses** are restricted to the respiratory tract, where they multiply and produce local disease. These are known as respiratory viruses. Some of these viruses **multiply locally** to initiate a silent local infection. This is followed by lymphatic or hematogenous spread to other organs where more extensive multiplication takes place before systemic illness is manifested. Smallpox and chickenpox are examples of such systemic diseases in which the portal of entry is the respiratory tract.
2. The **alimentary tract** is the next most important route of entry for viruses. **Enteroviruses, adenoviruses, reoviruses, some hepatitis viruses** and viruses responsible for gastroenteritis can cause intestinal infection.

Some of these, such as rotavirus, remain confined to the gut, causing local disease. Others, such as poliovirus, after initial multiplication locally, are transported to other sites for further multiplication and subsequent spread to the target organs.

All enveloped viruses are destroyed by bile.

3. On the s**kin**, only a few viruses produce local lesions. Papilloma, vaccinia, cowpox, molluscum contagiosum and orf are viruses that

produce **dermal lesions at the site of entry**. Viruses enter the skin through abrasions (papillomavirus), insect bites (arboviruses), animal bites (rabies) or injections (type B hepatitis). Systemic spread occurs through the lymphatics or blood. The rabies virus travels along the nerves to the spinal cord or brain. Skin lesions of exanthematous viral diseases are secondary to systemic infection.

4. The **conjunctiva** also may act as a portal of entry for viruses. This may lead to local disease (adenovirus) or to systemic spread (measles).
5. Some viruses may enter through the **genital tract** or other sites of **sexual contact** (HIV, genital herpes and human papillomavirus).
6. **Congenital infection** may occur at any stage of conception, from the development of the ovum up to birth. In acute systemic infections, congenital infection usually leads to fetal death and abortion. Rubella and cytomegalovirus produce congenital anomalies or severe neonatal disease.

Spread of Viruses in the Body

Viruses multiply initially at the site of entry and proceed along the lymphatics to the local nodes (Fig. 41.2). After multiplication in the lymph nodes, the virus enters the bloodstream (**primary viremia**) and is transported to the spleen and liver, which act as the 'central foci' for viral multiplication. After extensive multiplication in the central foci, they invade the bloodstream (**secondary viremia**). This heralds the onset of clinical symptoms (the prodromal phase in eruptive fevers). The virus reaches the target organ (e.g., skin in exanthematous fevers) through the bloodstream. Multiplication in the target sites produces the distinctive lesions. With minor modifications, this model holds good for most systemic virus diseases. As the virus matures, the infection spreads by a process of **shedding** through body fluids and secretions/excretions. This enables the dissemination of the virus through aerosols, direct contact or through fomites.

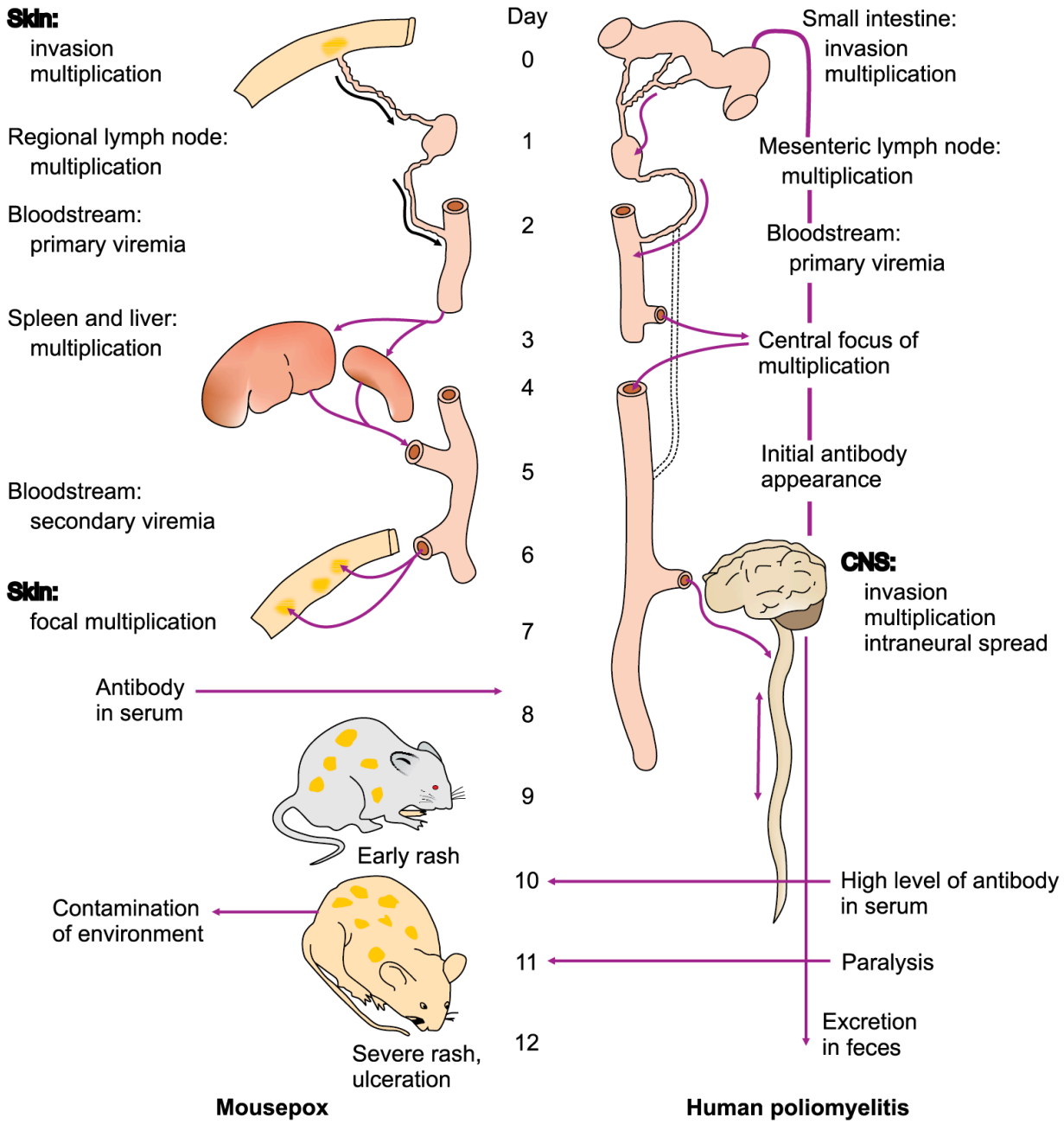


Fig. 41.2 Schematic illustrations of the pathogenesis of mousepox and poliomyelitis

Significance of the Incubation Period

The incubation period represents the time taken for the virus to spread from the site of entry to the organs of viral multiplication and then to the target organs. Where the site of entry and site of lesion are the same, the incubation period is short—one to three days, as in the respiratory tract and the gut. In systemic diseases with lesions in remote target sites, the

incubation period is longer—10–20 days, as in chickenpox or poliomyelitis. In arbovirus diseases, as in yellow fever or dengue, the incubation period may be shorter (5–6 days), probably because the virus is introduced directly into the bloodstream by the insect vectors. The incubation period for hepatitis B may be 2–6 months and in slow viral infections, many years. Papillomas and molluscum contagiosum have long incubation periods, probably because the viruses multiply slowly.

HOST RESPONSE TO VIRAL INFECTIONS

The outcome of a virus infection is influenced by the virulence of the infecting strain and the resistance offered by the host.

Immunological Responses

Humoral

IgG, IgM and IgA play a major role in protective immunity. While IgG and IgM play a critical role in blood and tissue immunity, IgA is important in mucosal immunity.

Antibodies may act as follows:

- By preventing adsorption of the virus to cell receptors
- By causing enhanced virus degradation
- By preventing the release of the progeny virus from infected cells

Complement acts in conjunction with antibodies in causing surface damage to enveloped virions by cytolysis of virus-infected cells.

Antibodies synthesised against antigens within the virus are non-neutralising. Antibodies to surface antigens vary in their neutralising ability. For example, antihemagglutinin antibody of influenza virus can neutralise infectivity, whereas antineuraminidase cannot. Humoral antibodies may sometimes contribute to the pathogenesis due to antibody-dependent enhanced activity as mentioned below:

- They may produce complement-dependent injury to cells or induced immune complex type of tissue injury.

- Enhanced severity of respiratory syncytial viral infection in early infancy is believed to be due to the presence of passively acquired maternal antibodies. In older children who have no antibody, the virus causes a milder disease.
- Pathogenesis of some viral hemorrhagic fevers is immune thrombocytopenia, e.g., dengue shock syndrome and (DSS) or dengue hemorrhagic fever (DHF).
- Most extrahepatic lesions in hepatitis B infection are due to damage caused by immune complexes.

Cell-mediated

Cell-mediated immunity is important as viruses have obligate intracellular existence. Individuals with deficient cellular immunity show heightened susceptibility to infection by the herpes, pox and measles viruses. Cell mediated immunity is considered to play a major role in recovery from viral infections. However, in some viral infections, cell-mediated immunity may contribute to tissue damage, as in lymphocytic choriomeningitis in mice.

Suppression

Some viral infections cause suppression of the immune response. Measles infection induces a temporary depression of delayed hypersensitivity to tuberculin. HIV suppresses the immune system by infecting the $CD4^+$ helper T cell.

In general, viral infections induce immunity to re-infection, which may be lifelong. Apparent exceptions like the common cold and influenza are not due to lack of immunity but due to re-infection caused by antigenically different viruses.

Non-immunological Responses

Body temperature

Fever may act as a natural defence mechanism against viral infections as most viruses are inhibited by temperatures above 39°C. An exception is herpes simplex, which is usually reactivated by fever to produce 'fever blisters' or Herpes febrilis.

Fever blisters are the frequent accompaniment of fevers caused by pneumococci, streptococci,

influenza virus and malaria but for some unknown reason, they are very rare in other fevers (typhoid and tuberculosis).

Hormones

Corticosteroid administration enhances most viral infections, perhaps due to its depression of the immune response and inhibition of interferon synthesis. Mild infections such as varicella and vaccinia may be lethal for patients on cortisone. Heavy doses of steroids in the treatment of herpetic keratoconjunctivitis may cause blindness. The particularly severe course of many viral infections in pregnancy may be related to the associated hormonal changes.

Malnutrition

Some viral infections such as measles produce a much higher incidence of complications and a higher case fatality rate in malnourished children than in well-fed patients.

Age

Most viral infections are commoner and more dangerous at the two extremes of age.

Interferon

It is a regulatory peptide belonging to the class of **cytokines**. It is produced by activated T cells as a natural defence mechanism against viral infections.

Interferons are a family of host-coded proteins which are α-helical cytokines produced in response to specific extracellular agents like viruses, tumour cells and other biomolecules by stimulation of toll-like receptors (TLRs). Interferon by itself has no direct action on viruses but acts on other cells of the same species, rendering them refractory to viral infection. On exposure to interferon, cells produce a protein (**translation inhibiting protein, TIP**), which includes a protein kinase, an oligonucleotide synthetase and an RNase. This protein selectively inhibits the translation of viral mRNA into viral proteins, without affecting cellular mRNA. It has also been suggested that the inhibition of viral transcription may also be responsible for the antiviral activity of the interferon.

Interferons are species-specific. They can protect only cells of the same or related species against viral infections. The interferon induced by one virus

(or even by non-viral inducers) can confer protection against infection by the same or unrelated viruses. However, **viruses vary in their susceptibility to interferons** and also vary in their capacity to induce interferons. RNA viruses are better inducers than DNA viruses. Examples of potent inducers are togaviruses, vesicular stomatitis virus, Sendai virus and Newcastle disease virus (NDV).

Interferon synthesis begins within about an hour of induction and reaches high levels in 6–12 hours. The promptness of interferon induction—much quicker than the antibody response—suggests that **interferons may play a primary role in host defence against viral infections.**

Types of interferons

Based on antigenic character, cell of origin and other properties, interferons have been classified into three types: **alpha, beta** and **gamma**.

- **Alpha interferon (IFN-α)** is produced by leucocytes following induction by suitable viruses. It is a non-glycosylated protein. At least **16 antigenic subtypes have been identified**.
- **Beta interferon (IFN-β)** is produced by fibroblasts and epithelial cells following stimulation by viruses or polynucleotides. It is a glycoprotein.
- **Gamma interferon (IFN-γ)** is produced by T cells on stimulation by antigens or mitogens. It is a glycoprotein.

Clinical uses

Many properties of interferons make them ideal candidates for use in the prophylaxis and treatment of viral infections; they are non-toxic, non-antigenic, diffuse freely in the body and have a wide spectrum of antiviral activity. Human interferon is available for therapeutic use. Limited success has been reported against generalised herpes infection in immunocompromised hosts and against hepatitis B and C infections. Interferons have been used as anticancer agents, particularly in lymphomas.

Interferons also inhibit cell growth and proliferation and DNA and protein synthesis, and increase expression of MHC antigens on cell surfaces.

Immunoregulatory effects of interferons These include enhanced

cytotoxic activity of NK, K and T cells, activation of macrophage cytocidal activity, modulation of antibody formation, activation of suppressor T cells and suppression of DTH.

LABORATORY DIAGNOSIS OF VIRAL DISEASES

Diagnosis of viral infections was limited in the past due to the technical difficulties in virus isolation, identification, and time required for the final identification, by which time the patient would either have recovered or succumbed. Also, since there was no treatment for viral infections, specific diagnosis of a viral infection did not impact management.

However, this has changed in recent times. Diagnostic virology has rapidly come into the routine scope of clinical laboratories for several reasons:

i) Technological developments in the area of understanding the process of nucleic acid synthesis and production have been helpful.
ii) Development of antiviral drugs and their therapeutic use has made it necessary to diagnose viral infections for specific treatment.
iii) Diagnosis of viral infections causing immunosuppression is necessary in order to provide prophylactic therapy.
iv) Follow-up and monitoring of chronic viral infections like HIV and HCV (hepatitis C virus) by molecular methods is now possible.
v) Preventive care has made it necessary to screen populations at risk for some of these viral infections, e.g., HIV, hepatitis B and C.
vi) It is essential for the detection, prevention and control of epidemics and pandemics.
vii) It is necessary to control outbreaks by vaccination of vulnerable population, and estimating protective antibodies in them.

Specimen

Successful diagnosis of viral infections depends as much on the awareness of the physician as on the capability of the virus laboratory. The appropriate specimens should be collected from patients, preserved and transported to the laboratory in the proper manner along with pertinent clinical and epidemiological information (Table 41.2).

Table 41.2 Types of specimens to be sent for virus diagnosis

System	Specimens required[1]		
	For isolation	For direct examination[2]	For serology
Respiratory system; nasopharyngeal	Throat swab, nasopharyngeal and oropharyngeal swabs throat washings (EM) aspirates	Nasopharyngeal aspirate (IF)	Paired sera
Central nervous system	Feces, blood (for arbovirus isolation) CSF (brain biopsy, throat swab, rectal swab)	Brain biopsy (IF and EM) CSF (EM and IF)	Paired sera
Cardiovascular system	Feces	Nil	Paired sera
Skin	Macular/papular scrapings, vesicular/pustular fluid, ulcer scrapings, crust, feces, throat swab	Vesicular/pustular fluid (EM and ID) Ulcer scrapings (EM), crusts (EM and ID)	Paired sera
Eye	Conjunctival scrapings or swabs	Conjunctival scrapings, as smears on microscope slides (LM and IF)[3]	Paired sera
Liver	Blood (for yellow fever)	Serum (feces)	Serum
General; congenital infections	Throat swab (for products of conception)	Nil	Single sera (mother and baby)
General; fever of unknown origin (FUO)	Heparinised blood (arbovirus and arenavirus infections) throat swabs, feces (fresh urine)	Nil	Paired sera

Microscopy

Virus elementary bodies can be demonstrated by the examination of stained smears under a light microscope. Inclusion bodies may be visualised by specific staining methods in several viral infections. Demonstration of the

inclusion body (Negri bodies) on Seller's stain is a routine method for the diagnosis of rabies in dogs. Detection of the virus by **electron microscopy** is being used increasingly. **Immunoelectron microscopy** has been used to detect Nipah viruses from tissue samples.

A fluorescent microscope can be used for the microscopic diagnosis of rabies by **fluorescent antibody** techniques. The use of direct and indirect fluorescent antibody techniques is used to examine material from lesions, as well as for the early demonstration of viral antigen in tissue cultures inoculated with specimens.

Demonstration of Virus Antigen

In cases where the virus antigen is abundant in the lesions, its demonstration by serological methods like immunofluorescence and enzyme immune assays act as rapid methods of diagnosis. Highly sensitive serological tests such as enzyme-linked immunosorbent assays and chemiluminescence assays have found wide application in diagnostic virology for the detection of viral antigens in clinical samples.

Isolation of Virus

Specimens for virus isolation are collected aseptically and transported with least delay for further processing. As most viruses are heat-labile, **refrigeration is essential** during transport (for most specimens). The methods used for isolation depend on the virus sought. In general, they consist of tissue culture, embryonated egg inoculation, and animal inoculation via various routes. This is done after the specimen is processed to remove bacterial contaminants. The isolates are identified by neutralisation or other suitable serological procedures. It has to be emphasised that the mere recovery of a virus from a patient cannot confirm that it is the causative agent of the patient's illness. Many viruses (e.g., adenoviruses and enteroviruses) are frequently found in healthy individuals.

CULTIVATION OF VIRUSES

As viruses are obligate intracellular parasites, they cannot be grown on any inanimate culture medium. Three methods are employed for the cultivation of viruses: inoculation into animals, embryonated eggs or tissue cultures.

Animal Inoculation

The earliest method for the cultivation of viruses causing human diseases was inoculation into human volunteers—humans were later replaced by various laboratory animals. Mice are the most widely used animals in virology.

Infant (suckling) mice are very susceptible to coxsackie and arboviruses, many of which do not grow in any other system. Mice may be inoculated by several routes: intracerebral, subcutaneous, intraperitoneal or intranasal.

Other animals such as **guinea pigs, rabbits** and **ferrets** are used to isolate viruses through inoculation via various routes.

The growth of the virus in inoculated animals may be indicated by:

- Death
- Disease
- Visible lesions

Serial blind passages may sometimes be necessary before evidence of virus growth can be obtained.

> The disadvantages of animal inoculation are that immunity may interfere with viral growth and that animals often harbour latent viruses. Animal inoculation is also used for the study of pathogenesis, immune response, epidemiology and oncogenesis.

Embryonated Eggs

Embryonated hen's egg was first used for the cultivation of viruses by Goodpasture (1931) and the method was further developed by Burnet. The embryonated egg offers several sites for the cultivation of viruses (Fig. 41.3).

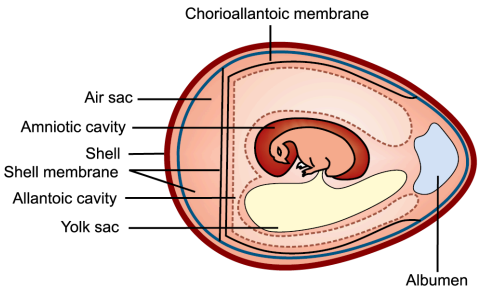


Fig. 41.3 10-day-old embryonated hen's egg

Inoculation on the **chorioallantoic membrane (CAM)** produces visible

lesions (pocks). Different viruses have different pock morphology. Under optimal conditions, each infectious virus particle can form one pock. Pock counting, therefore, can be used for the assay of pock-forming viruses such as variola or vaccinia.

Inoculation into the **allantoic cavity** provides a rich yield of influenza and some paramyxoviruses.

Inoculation into the **amniotic sac** is employed for the primary isolation of the influenza virus.

Yolk sac inoculation is used for the cultivation of some viruses, chlamydiae and rickettsiae.

Allantoic inoculation is employed for growing the influenza virus for vaccine production. Other chick embryo vaccines in routine use are the yellow fever **(17D strain)** and rabies (**Flury strain**) vaccines. Duck eggs are bigger and have a longer incubation period than hen's eggs. They, therefore, provide a better yield of rabies virus and were used for the preparation of the inactivated non-neural rabies vaccine.

Tissue Culture

Human virus has been grown in tissue culture (Fig. 41.4).

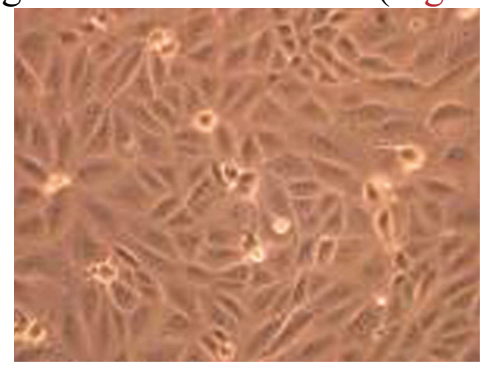


Fig. 41.4 Normal Vero cell monolayer

Types of tissue cultures

1. Organ culture

Small bits of organs can be maintained in vitro for days and weeks, preserving their original architecture and function. Organ cultures are useful for the isolation of some viruses which appear to be highly specialised parasites of certain organs. For example, the tracheal ring organ culture is employed for the isolation of coronavirus, a respiratory pathogen.

2. Explant culture

Fragments of minced tissue can be grown as '**explants**' embedded in plasma clots. They may also be cultivated in suspension. This was originally known as 'tissue culture'. This method is now seldom employed in virology. Adenoid tissue explant cultures were used for the isolation of adenoviruses.

3. Cell culture

This type of culture is routinely employed for growing viruses. Tissues are dissociated into component cells by the action of proteolytic enzymes such as trypsin and mechanical shaking. The cells are washed, counted and suspended in a growth medium. The essential constituents of the **growth medium** are physiologic amounts of essential amino acids and vitamins, salts, glucose and a buffering system generally consisting of bicarbonate in equilibrium with the atmosphere containing about **5% carbon dioxide**. This is supplemented with up to **5% calf or fetal calf serum**. Antibiotics are added to prevent bacterial contaminants and phenol red is used as an indicator. Such media will enable most cell types to multiply with a division time of 24–48 hours. The cells adhere to the glass surface and on incubation, divide to form a confluent monolayer sheet of cells covering the surface within about a week.

Cell culture tubes may be incubated in a sloped horizontal position, either as '**stationary culture**' or may be rolled in special '**roller drums**' to provide better aeration. Some fastidious viruses grow only in such roller cultures.

Based on their origin, chromosomal characters and the number of generations through which they can be maintained, cell cultures are classified into three types (Table 41.3):

Table 41.3 Some cell cultures in common use

1. Primary cell cultures	
Rhesus monkey kidney cell culture	
Human amnion cell culture	
Chick embryo fibroblast cell culture	
2. Diploid cell strains	**Examples**

WI-38 (Wistar Institute-38)	Human embryonic lung cell strain
MRC-5 (Medical Research Cell Strain-5)	Fibroblast from lung tissue
3. Continuous cell lines	**Examples**
HeLa	Human carcinoma of cervix cell line
HEp-2	Human epithelioma of larynx cell line
KB	Human carcinoma of nasopharynx cell line
McCoy	Human synovial carcinoma cell line
Detroit 6	Sternal marrow cell line
Chang C/I/L/K	Human conjunctiva (C) intestine (I), liver (L) and kidney (K) cell lines
Vero	Vervet monkey kidney cell line
BHK-21	Baby hamster kidney cell line

i) ***Primary cell cultures*** These are normal cells freshly taken from the body and cultured. They are capable of only limited growth in culture and cannot be maintained in serial culture. Common examples of primary cell cultures are monkey kidney, human embryonic kidney, human amnion and chick embryo cell cultures. Primary cell cultures are useful for the **isolation of viruses** and their cultivation for **vaccine production**.

ii) ***Diploid cell strains*** These are cells of a single type that retain their original diploid chromosome number and karyotype during serial subcultivation for a limited number of times. After about fifty serial passages, they undergo 'senescence'. Diploid strains developed from **human fibroblasts** are susceptible to a wide range of human viruses and are very useful for the isolation of some fastidious pathogens. They are also used for the **production of viral vaccines.**

iii) ***Continuous cell lines*** These are cells of a single type, usually derived from cancer cells, which are capable of continuous serial

cultivation indefinitely. Standard **cell lines derived from human cancers** such as HeLa, HEp-2 and KB cell lines have been used in laboratories across the world for many years. These cell lines may be maintained by serial subcultivation or stored in the cold (–70°C) for use when necessary. Some cell lines are now permitted to be used for vaccine manufacture, for example, Vero cell for rabies vaccine.

Detection of viral growth in infected tissues is seen as inclusions.

Detection of virus growth in cell cultures Virus growth in cell lines may be detected by any one or more of the following methods:

i) ***Cytopathic effect*** Many viruses cause morphological changes in the cultured cells in which they grow. These changes can be readily observed by microscopic examination of the cultures. These changes are known as '**cytopathic effects**' **(CPE)** and the viruses causing CPE are called '**cytopathogenic viruses**'. The CPE produced by different groups of viruses are characteristic and help in the presumptive identification of virus isolates. For example, enteroviruses produce rapid CPE with crenation of cells and degeneration of the entire cell sheet; the measles virus produces syncytium formation (Fig. 41.5); the herpes virus causes discrete focal degeneration; the adenovirus produces large granular clumps resembling bunches of grapes; and SV40 produces prominent cytoplasmic vacuolation.

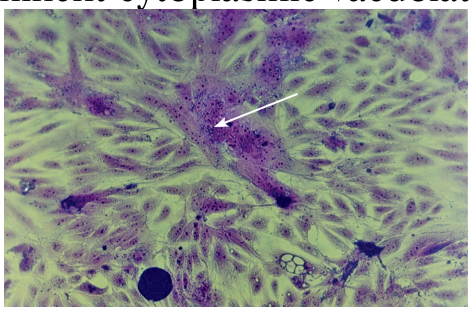


Fig. 41.5 Vervet monkey kidney cells (Vero cell line) infected with measles virus (Note syncytium formation, crystal violet stained ×100)

ii) ***Metabolic inhibition*** In normal cell cultures, the medium turns acidic due to cellular metabolism. When viruses grow in cell cultures, cell metabolism is inhibited and there is no acid production. This can be made out by the colour of the indicator (phenol red) incorporated in the medium.

iii) ***Hemadsorption*** When hemagglutinating viruses (such as influenza and parainfluenza viruses) grow in cell cultures, their presence can be indicated by the addition of guinea pig erythrocytes to the cultures. If the viruses are multiplying in the culture, the erythrocytes will adsorb onto the surface of the cells. This is known as 'hemadsorption'.

iv) ***Interference*** The growth of a non-cytopathogenic virus in cell culture can be tested by the subsequent challenge with a known cytopathogenic virus. The growth of the first will inhibit infection by the second virus by interference.

v) ***Transformation*** Tumour-forming (oncogenic) viruses induce cell 'transformation' and loss of contact inhibition, so that growth appears in a piled-up fashion, producing 'microtumours'.

vi) ***Immunofluorescence*** Cells from virus-infected cultures can be stained by fluorescent conjugated antiserum and examined under the UV microscope for the presence of virus antigen. This gives positive results earlier than other methods and, therefore, finds wide application in diagnostic virology.

VIRAL ASSAY

The virus content of a specimen can be assayed in two ways—either with reference to the total virus particles or with reference to the infectious virions only. Two methods are used for total particle enumeration:

1. By simple negative staining, the virus particles in a suspension can be counted directly under the **electron microscope**. The virus suspension can be mixed with a known concentration of latex particles. The ratio between the virus and latex particles under the electron microscope gives an indication of the virus count.
2. With hemagglutinating viruses, a convenient method of quantitation is the determination of **hemagglutination titres**. Hemagglutination is not a very sensitive indicator of the presence of small amounts of virus particles. Approximately 10^7 influenza virions are required to produce macroscopic agglutination of a convenient quantity of chicken erythrocytes (0.5 mL of 0.5% suspension). However, because of its simplicity, hemagglutination is a very convenient method of virus assay.

ASSAY OF INFECTIVITY

Two types of infectivity assays can be carried out: quantitative and quantal. Quantitative assays measure the actual number of infectious particles in the inoculum, while quantal assays only indicate the presence or absence of infectious viruses.

Quantitative Assays

The quantitative infectivity assay of viruses is similar to the estimation of bacterial viable counts by colony counting. Two methods are available:

1. ***Plaque assay*** This is a method to detect virus growth in cell lines. A viral suspension is added to a monolayer of cultured cells in a bottle or petri dish, and after allowing time for absorption, the medium is removed and replaced with a solid agar gel to ensure that the spread of progeny virions is confined to the immediate vicinity of the infected cells. In this system, each infectious viral particle gives rise to a localised focus of infected cells that can be seen with the unaided eye. Such foci are known as 'plaques' and each plaque indicates an infectious virus. Some viruses that are transmitted directly from cell to cell (e.g., herpesvirus) may form plaques even without an agar overlay. Oncogenic viruses produce cell transformation which can be seen as microtumours. Hence they can be enumerated by the transformation assay.
2. ***Pock assay*** In this method, viruses that form pocks on CAM (e.g., vaccinia) can be assayed by counting the number of pocks formed on CAM by the appropriate inocula of virus.

Quantal Assays

The titre (serial dilutions of virus suspensions are expressed in titres) is usually expressed as '50 per cent infectious dose' (ID_{50}) per mL, which indicates the highest dilution of the inoculum that would produce an effect in 50 per cent of animals, eggs or cell cultures inoculated.

VIRAL HEMAGGLUTINATION

A large number of viruses have since been shown to agglutinate

erythrocytes from different species. Hemagglutination by the influenza virus is **due to the presence of hemagglutinin spikes on the surface of the virus**. The influenza virus also carries on its surface another peplomer, the enzyme neuraminidase which acts on the receptor and destroys it. **Neuraminidase** is, therefore, called the **receptor destroying enzyme (RDE).** RDE is produced by many microbes including cholera vibrios, and is also present in many vertebrate cells. Destruction of the receptor leads to the reversal of hemagglutination and the release of the virus from the red cell surface. This is known as **elution.**

Viral hemagglutination is used in the detection and assay of influenza virus. When red cells are added to serial dilutions of a viral suspension, the highest dilution that produces hemagglutination provides the hemagglutination titre (Fig. 41.6).

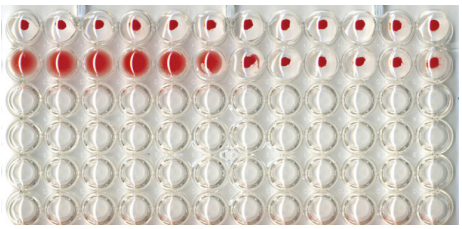


Fig. 41.6 Viral hemagglutination

Uses of hemagglutination
- To titrate killed influenza vaccines
- To detect antiviral antibody by hemagglutination inhibition (HAI) as hemagglutination is specifically inhibited by the antibody to the virus
- To purify and concentrate the virus

Elution **Viruses that possess neuraminidase on their surface will cause RBC to get detached from them, thereby reversing the agglutination. This phenomenon is known as elution.** This is found only in myxoviruses that possess neuraminidase. With other viruses, hemagglutination is stable.

In arboviruses, hemagglutination appears to be a reversible state of equilibrium between the virus and erythrocytes, which depends on pH and temperature. Poxviruses agglutinate red cells from only certain fowls. Table 41.4 shows specific RBCs which are agglutinated by different viruses.

Table 41.4 Characteristics of hemagglutination by viruses

Virus	Erythrocyte species and other conditions

Influenza virus	Fowl, human, guinea pig, others; elution at 37°C
Parainfluenza, mumps, NDV	Fowl, human, guinea pig, others; elution at 37°C; hemolysin present
Measles	Monkey, 37°C
Togavirus, several groups of arbovirus	Goose, pigeon, one-day-old chick; pH and temperature critical
Rubella	Goose, pigeon, one-day-old chick; 4°C
Enterovirus, some coxsackie and ECHO	Human; 4°C and 37°C
Rhinovirus, some serotypes	Sheep; 4°C
Rabies	Goose; 4°C, pH 6.2
Reovirus	Human; 37°C

SEROLOGICAL DIAGNOSIS

The demonstration of a **rise in titre of antibodies** to a virus during the course of a disease is strong evidence that it is the causative agent. It is essential to examine paired sera, for which samples are collected as follows:
i) Acute phase sample is collected early in the course of the disease.
ii) 'Convalescent' sample is collected 10–14 days later.

Examination of a single sample of serum for antibodies may not be meaningful except when IgM-specific tests are done. The serological techniques in general use are ELISA and other immunoassays, neutralisation, complement fixation and hemagglutination inhibition tests.

MOLECULAR DIAGNOSIS

The availability of molecular methods has transformed the diagnosis of viral diseases, enlarging the scope, sensitivity and specificity of such tests. In situ hybridisation using nucleic acid probes has been used to diagnose human papillomaviruses. Nucleic acid amplification techniques like PCR, reverse transcriptase PCR and real-time PCR have increased the sensitivity of the diagnostic assays. Following PCR, DNA or RNA sequencing of the product can be done. Molecular detection techniques have revolutionised

rapid identification and containment programmes for some fatal epidemics and pandemics like Ebola, Nipah, H1N1, SARS, MERS-CoV and COVID-19.

Key points

- Virus–host interactions can take place at the level of the cell, individual and community. At the cellular level, viral infection may cause a broad spectrum of effects, ranging from no apparent effects to rapid cell destruction.
- Many viruses produce alterations in the cytoplasmic membrane of the infected cells and cause fusion of adjacent cell membranes or the appearance of hemagglutinins on the surface of infected cells.
- The most characteristic histological feature in virus-infected cells is the appearance of inclusion bodies; these can be seen by light microscopy and may be acidophilic or basophilic and intracytoplasmic or intranuclear. The Negri body is an acidophilic, intracytoplasmic inclusion body in rabies virus.
- Depending on the clinical outcome, viral infections can be classified as subclinical (inapparent), clinical or overt (apparent) or latent infections which remain quiescent for long periods. Viruses enter the body through the respiratory tract, alimentary tract, skin, conjunctiva and the
  genital tract.
- The infecting virus usually spreads from the point of entry and multiplies in sites of selection. In systemic virus infections, there is entry, spread to the local lymph nodes, multiplication, entry into the bloodstream (primary viremia), spread to central liver and spleen and secondary viremia and finally, spread to the target organs.
- The incubation period is the time taken for the virus to spread from the site of entry to the organs of viral replication.
- Viruses are intracellular parasites; a cell infected by a virus may undergo immediate degeneration or destruction, proliferate and then undergo necrosis or not change at all, causing latent virus infections.
- Interferon is a protein that may be produced by a virus-infected cell or a sensitised lymphocyte encountering such a cell. Interferon alters cellular metabolism so that further assembly of viral particles is prevented.
- Specific humoral and cell-mediated immune responses may be observed in viral infections. Cell-mediated immune responses confer protection against viral diseases.
- Vaccination (active immunisation) against various viral diseases augments cell-mediated immunity. Overreaction of cell-mediated immune responses to viral infections may result in tissue damage (delayed hypersensitivity reactions).
- Several methods are used in the laboratory for the diagnosis of viral infections:

    - Viruses in infected specimens may be detected by electron microscopy. Light microscopy can be used to detect viral inclusion bodies in Giemsa-stained smears.
    - Specific viral antigens in the blood or body fluids of an infected individual can sometimes be detected.
- Cultivation of viruses can be done by inoculation into animals, embryonated hens' eggs or tissue culture, but may vary. Viruses can be cultivated in suckling mice (24–48 hours old), guinea pigs and rabbits.
- For cell culture, primary cell cultures, secondary and continuous cell lines—for example, HeLa, HEp-2, Vero and BHK-21—can be used.
- The growth of a virus in such cell cultures can be detected by tests that demonstrate cytopathic effect (CPE), metabolic inhibition, hemadsorption (HEA) and transformation and by immunofluorescence.
- The virus content of a specimen can be assayed either with reference to the total virus particles or to the infectious virions alone. Two types of infectivity assays can be performed: quantitative, which measure the actual number of infectious particles in the inoculum, and quantal, which only indicates the presence or absence of infectious viruses.
- Serological diagnosis using neutralisation, complement fixation, hemadsorption inhibition, immunofluorescence, enzyme immunoassays and radioimmunoassay is useful. IgM antibody or four-fold rise in IgG antibody levels in paired serum against a specific virus denotes an active infection.
- Diagnosis of viral infections by polymerase chain reaction (PCR) is a very sensitive technique that can detect a single molecule of viral DNA in a clinical specimen.

Questions

Essays

1. Describe the various routes of transmission of viruses and their pathogenesis.
2. Mention the need for laboratory diagnosis of viral infections. Describe the general procedure to diagnose these infections.

Short Answers

1. Detection of viral growth in cell lines
2. Tests employed to detect viral antibodies
3. Methods of cultivation of viruses
4. Cell cultures for virus isolation
5. Interference in viruses

Part IV: Virology

Chapter 42

Bacteriophages

Chapter navigation

- Morphology
- Life cycle
- Significance in medical microbiology
- Phage typing
- Phage therapy

INTRODUCTION

Bacteriophages are viruses that infect bacteria. They exist widely in nature in close association with bacteria. Phages can be readily isolated from feces, sewage and other natural sources of mixed bacterial growth. Early hopes that phages could be used in the treatment of bacterial infections have not been fulfilled. However, interest in phage therapy is now gaining momentum in response to rapidly emerging antibiotic resistance.

Historical perspective
Twort (1915) described a degenerative change in staphylococcal colonies, which could be transmitted serially by the application of culture filtrates from the original growth. D'Herelle (1917) observed that filtrates of feces cultures from dysentery patients induced transmissible lysis of a broth culture of a dysentery bacillus. He suggested that the lytic agent was a virus and gave it the name bacteriophage.

The **functions** of the phages of importance are as follows:

- Transmission of genetic information between bacteria is by **transduction**.
- The presence of phage genome integrated with bacterial chromosomes confers on bacteria certain properties by a process known as **phage conversion**.
- Phages have been used as **cloning vectors** in genetic manipulations.

- The presence of high concentrations of phage particles, up to 10^8 per mL in some natural waters, suggests that they may have a role in the **control of bacterial populations** in such environments.
- The specificity of the host range of phages is the basis of **phage typing methods**, by which bacteria can be identified and typed.
- **Phage therapy** is being considered as an alternative to antibiotic therapy in multidrug-resistant infections.

MORPHOLOGY

Bacteriophages that infect *E. coli,* called the **T-even phages** (T2, T4, T6), have been studied in detail and have traditionally served as the prototypes in describing the properties of bacteriophages. T-even phages have a complex morphology. They are tadpole-shaped, with a hexagonal head and a cylindrical tail.

The **head** consists of a tightly packed core of nucleic acid (double-stranded DNA) surrounded by a protein coat or capsid. The size of the head varies in different phages, from 28 nm to 100 nm.

The **tail** is composed of a hollow core, a contractile sheath surrounding the core and a terminal base plate which has prongs, tail fibres or both attached to it
(Fig. 42.1a and b).

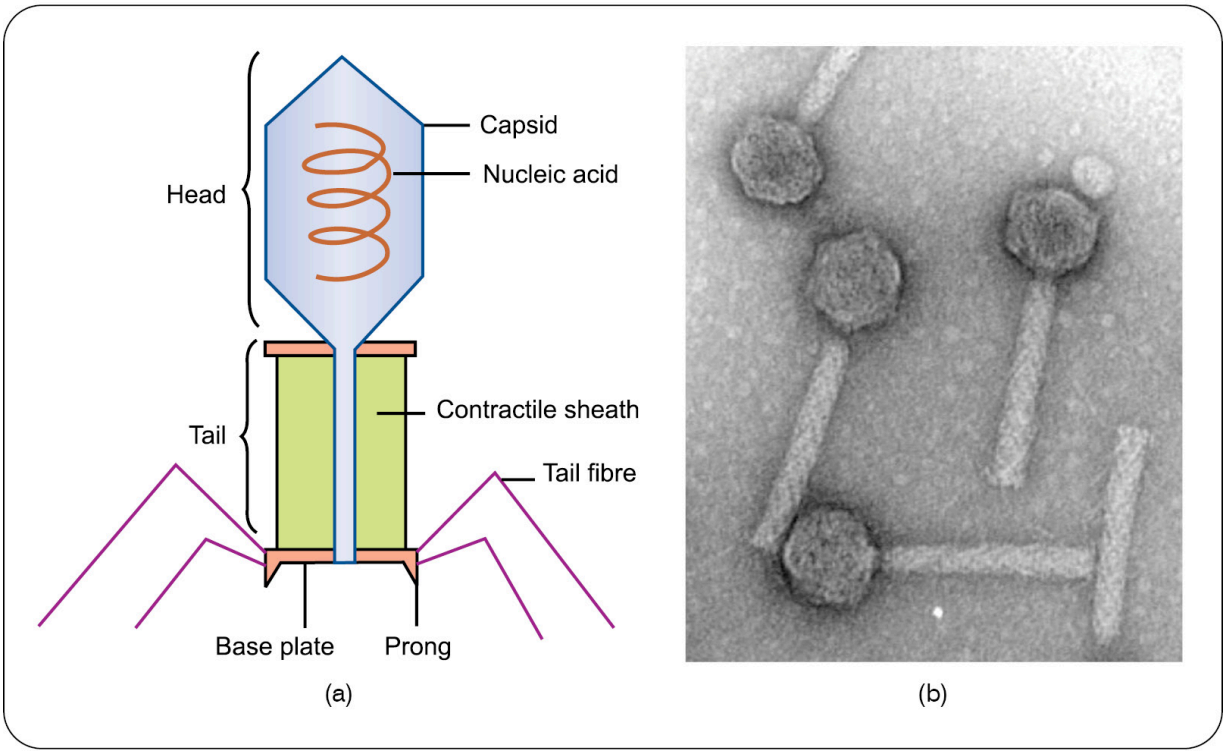


Fig. 42.1 (a) Morphology of a bacteriophage and (b) electron micrograph of bacteriophages

Though most bacteriophages have the morphology and structure described in the previous paragraph, phages that are spherical or filamentous and possess single-stranded DNA or RNA have also been identified.

LIFE CYCLE

Phages exhibit two different types of life cycles:

1. **Lytic or virulent cycle** Here, the intracellular multiplication of the phage culminates in the lysis of the host bacterium and the release of progeny virions.
2. **Lysogenic or the temperate cycle** The phage DNA becomes integrated with the bacterial genome, replicating synchronously with it, causing no harm to the host cell (Fig. 42.2).

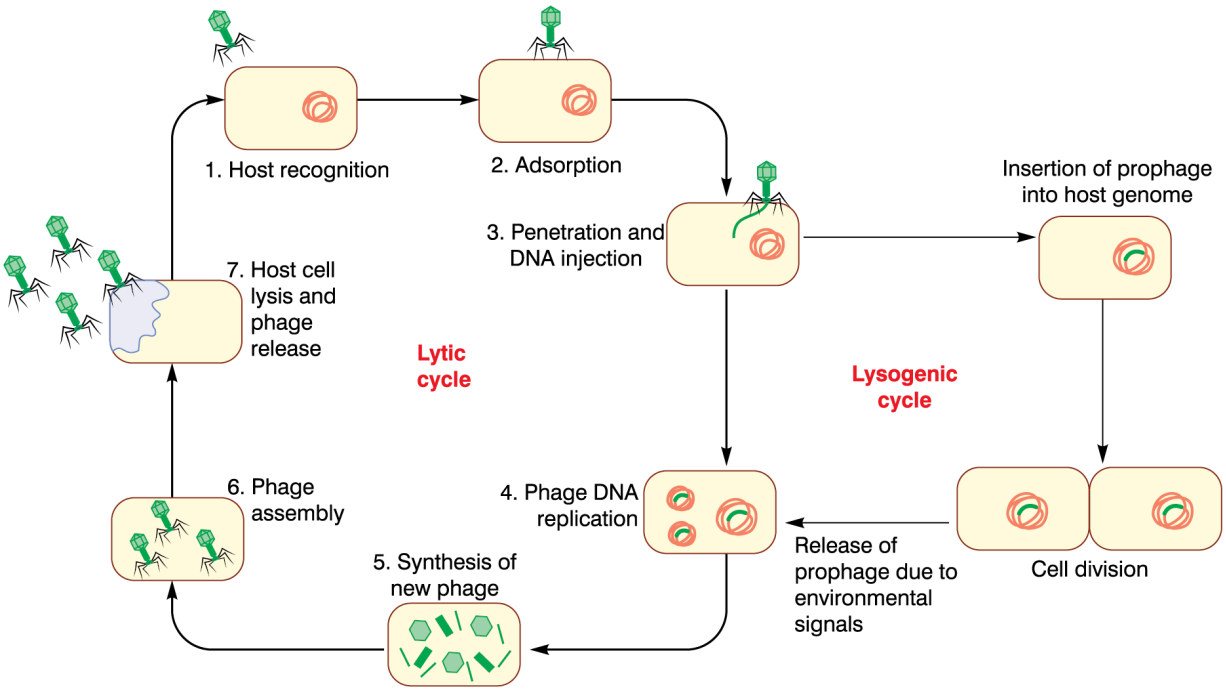

1. Host recognition
2. Adsorption
3. Penetration and DNA injection
Insertion of prophage into host genome
7. Host cell lysis and phage release
Lytic cycle
Lysogenic cycle
6. Phage assembly
4. Phage DNA replication
5. Synthesis of new phage
Release of prophage due to environmental signals
Cell division

Fig. 42.2 Lytic and lysogenic cycles of bacteriophages

1. Lytic Cycle

Replication of a virulent phage occurs in the following stages: adsorption, penetration, synthesis of phage components, assembly, maturation and release of progeny phage particles.

i) **Adsorption** Phage particles come into contact with bacterial cells by random collision and attach themselves to the surface of a susceptible bacterium by their tails. Adsorption depends on the presence of complementary chemical groups on the receptor sites of the bacterial surface and on the terminal base plate of the phage. It is a very rapid process, which is completed within minutes. Certain **co-factors**, like the cations, are necessary for adsorption. The bacterial receptor sites may be situated in different layers of the cell wall or on surface structures (such as the Vi antigen of *S.* Typhi) or appendages (flagella or sex pili). In vitro infection by direct injection of phage DNA can be achieved even in bacterial strains that are not susceptible to infection by the complete phage. The infection of a bacterium by the naked phage nucleic acid is known as **transfection**.

ii) **Penetration** The next step is the penetration of the phage nucleic acid into the bacterial cell. The process of penetration resembles injection

through a syringe. The base plate and tail fibres are held firmly against the cell, causing the hollow core to pierce through the cell wall. The contractile tail sheath acts like a muscle and derives its energy from a small amount of adenosine triphosphate present on the tail of the phage. The phage DNA is injected into the bacterial body through the hollow core. Penetration may be facilitated by the presence of lysozyme on the phage tail, which produces a hole on the bacterial wall for the entry of the phage core. After penetration, the empty head and tail of the phage remain outside the bacterium as a shell or 'ghost'.

When bacteria are mixed with phage particles at high multiplicity (high dose of phages), multiple holes are produced on the cell, with the consequent leakage of cell contents. Bacterial lysis occurs without viral multiplication. This is known as **lysis from without**.

iii) **Synthesis** Immediately after penetration, the synthesis of phages starts. The first products to be synthesised (called **early proteins**) are the enzymes necessary for the building of the complex molecules peculiar to the phage. Subsequently, **late proteins** appear, which include the protein subunits of the phage head and tail. During this period, the synthesis of bacterial protein, DNA and RNA ceases.

iv) **Maturation** The phage DNA, head protein and tail protein are synthesised separately in the bacterial cell. The DNA is condensed into a compact polyhedron and '**packaged**' into the head and, finally, the tail structures are added.

iv) **Release (lytic cycle)** This refers to the release of the mature progeny phages. During the replication of the phage, the bacterial cell wall is weakened by phage enzymes which cause the lysis of the cell.

Eclipse phase This is the interval between the entry of the phage nucleic acid into the bacterial cell and the appearance of the first infectious **intracellular** phage particle.

Latent period The interval between the infection of a bacterial cell and the first release of infectious phage particles is called the latent phase. As the replication rate increases, the number of phages increase. The average yield of progeny phages per infected bacterial cell is known as the **burst size**. The cycle of infection established with a single phage per cell can be plotted on a graph and this is known as the **one-step curve** (Fig. 42.3).

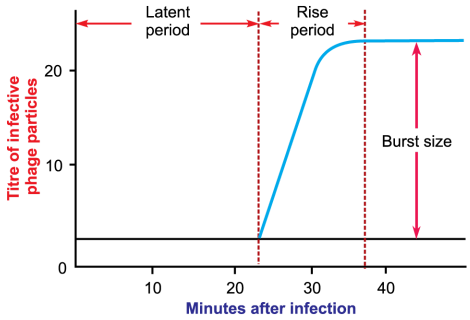


Fig. 42.3 One-step growth curve of bacteriophage

2. Lysogenic Cycle (Temperate Cycle)

Unlike virulent phages which produce lysis of the host cell, temperate phages enter into **a symbiotic relationship** with their host cells without destroying them (temperate cycle). The viral nucleic acid becomes integrated with the bacterial chromosome. The integrated phage nucleic acid is known as the **prophage**. The prophage replicates synchronously with the host chromosome. This phenomenon is called **lysogeny** and a bacterium that carries a prophage within its genome is called a **lysogenic bacterium.**

The prophage confers certain new properties on the lysogenic bacterium. This is known as lysogenic conversion or **phage conversion**. This is due to the synthesis of new proteins coded for by prophage DNA. An example is toxin production in *C. diphtheriae* by the **beta** prophage. Elimination of the prophage results in the loss of the toxigenicity of the bacillus.

UV rays, hydrogen peroxide and nitrogen mustard convert a lysogenic cycle to a lytic cycle. A lysogenic bacterium is resistant to re-infection by the same or related phages. This is known as **superinfection immunity**.

TRANSMISSION OF GENETIC INFORMATION

Bacteriophages may act as carriers of genes from one bacterium to another by **transduction,** which is of two types:

i) **Restricted transduction,** in which only bacterial genes contiguous to the prophage are transmitted. For example, transduction by the prophage lambda in *E. coli* K12 transfers only the gal^+ gene (determining fermentation of galactose), which is the bacterial gene contiguous to the prophage.

ii) **Generalised transduction,** in which any bacterial gene may be transferred.

Transduction has been demonstrated in many genera of bacteria and is one of the most important mechanisms of genetic exchange among bacteria in nature. **Plasmid-mediated drug resistance in staphylococci** is an example of a medically important property that is transmitted by transduction.

Significance of phages in medical microbiology

1. ***Phage assay*** When a phage is applied on the lawn culture of a susceptible bacterium, areas of clearing develop after incubation. These zones of lysis are called **plaques.** The size, shape and nature of plaques are characteristic for different phages. Under optimum conditions, a single phage particle is capable of producing one plaque. Plaque assay can be employed for titrating the number of viable phages in a preparation.
2. ***Phage typing*** The specificity of phage–bacterium interaction is made use of in the **identification and typing of bacteria**. Phages exhibit different degrees of host specificity. Some phages possess wide host ranges, spanning many bacterial genera, while others have a narrow range, limited to certain strains of bacteria only. With some phages, serial passage in a strain of bacterium makes them specific for that strain and related strains (adaptation of host range).

Phages may lyse:

- all the members of a bacterial genus (genus-specific bacteriophage for *Salmonella*),
- all the members of a species (specific bacteriophage for *B. anthracis*) or
- all the members of a biotype or subspecies (Mukherjee's phage IV, which lyses all strains of classical *V. cholerae* but not *V. cholerae* biotype El Tor) (Fig. 42.4).

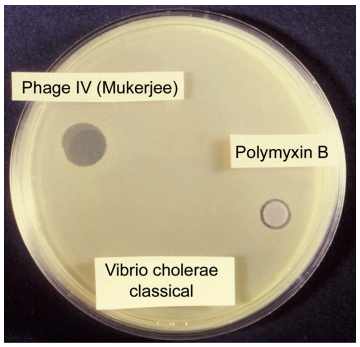


Fig. 42.4 Mukerjee's phage IV lysis of classical *V. cholerae*
(*Source:* Public Health Image Library, ID 3915 / CDC)

Other uses of phage typing are:

1. Intraspecies typing of bacteria

a) Phage typing of *S.*Typhi—adapted phages, active only against fresh isolates possessing the Vi antigen, are used for phage typing of the typhoid bacilli.

b) Staphylococcal phage typing is a pattern method using a set of standard phages. A strain of *Staphylococcus* may be lysed by a number of phages and the phage type of a strain is designated by the numbers of the different phages that lyse it. As lysis is influenced by the dose of infection, phage preparations used for typing should be standardised by titration.

2. Routine test dose (RTD) Titration is carried out by applying serial dilutions of the phage preparation on a lawn culture of a susceptible strain and observing the lysis after incubation. The highest dilution of the phage preparation that produces confluent lysis is known as the routine test dose.

PHAGE THERAPY

Renewed interest in bacteriophage research is being noted in recent years to study the diagnostic and therapeutic use of bacteriophages. Though phage therapy using bacteriophages was in use for over a century in some parts of the world, it was not employed in the western world. More recently, with the increase in the number of multidrug-resistant organisms and the failure of antibiotics, there has been renewed interest in using bacteriophages to treat infections. Conventionally, phage therapy relies on the use of naturally occurring bacteriophages that infect and lyse bacteria at the site of infection. The use of bioengineered phages resulting from biotechnological advances have expanded the repertoire through novel strategies of using whole phages or purified phage lytic proteins. Several successes have demonstrated the potential of using bacteriophages as an alternative or a supplement to antibiotic treatment.

Key points

- A bacteriophage is a type of virus that specifically infects bacteria and fungi.
- Bacteriophages exhibit two different types of life cycles:
    - **Lytic** (virulent) cycle, where intracellular multiplication of the phage ends in the lysis of the host bacterium and the release of progeny virions.
    - **Lysogenic** (temperate) cycle, where phage DNA becomes incorporated within the bacterial genome and then replicates synchronously with it, causing no harm to the host cell.
- The lytic cycle replication allows the bacteriophage to proceed through the stages of adsorption (attachment to the host cell), penetration of the nucleic acid into the cell, synthesis of phage components, assembly of phage components and maturation and release of progeny phage particles from the cell.
- Phages play an important role in the transmission of genetic information between bacteria by the process of transduction. They can carry antibiotic resistance genes or virulence genes. Bacteria can be typed by phage typing methods for epidemiological purposes.

Questions

Essay

1. Describe the lytic cycle of bacteriophages.

Short Answers

1. Structure of a bacteriophage (draw and describe)
2. Phage typing (definition)
3. Lysogeny (definition)
4. Uses of bacteriophages

Part IV: Virology

CHAPTER 43

Herpesviruses

Chapter navigation

- Herpes simplex
- Varicella-zoster
- Cytomegalovirus
- Epstein–Barr virus
- Human herpesvirus 8
(Kaposi's sarcoma-associated herpesvirus)

INTRODUCTION

The herpesvirus family contains over a hundred species of **enveloped DNA viruses t**hat affect humans and animals. They are characterised by their ability to establish **latent infections**, enabling the virus to persist indefinitely within infected hosts and to undergo **periodic reactivation.**

Morphology

The herpesvirus capsid is icosahedral, composed of 162 capsomers, enclosing the core containing the linear double-stranded DNA genome. The nucleocapsid is surrounded by the lipid envelope derived from the modified host cell nuclear membrane. The envelope carries surface spikes, about 8 nm long (Fig. 43.1). Between the envelope and the capsid is an amorphous structure called the tegument, containing several proteins. The enveloped virion measures about 200 nm and the naked virion, about 100 nm in diameter.

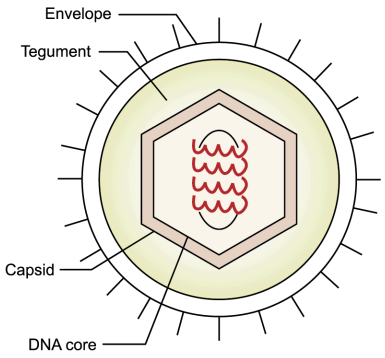


Fig. 43.1 Herpes simplex virus

Replication

Herpesviruses replicate in the host cell nucleus. They form **Cowdry type A intranuclear (Lipschutz) inclusion bodies.**

Resistance

Like other enveloped viruses, herpesviruses are susceptible to fat solvents like alcohol, ether, chloroform and bile salts. They are heat-labile and must be stored at –70°C.

Classification

Eight different types of herpesviruses are known to cause human infections. They have been officially termed **human herpesviruses** (Table 43.1).

Table 43.1 Classification and biological characters of important herpesviruses causing infection in humans

Viruses	Biological characters
Herpes simplex (HSV-1) (HSV-2) Varicella-zoster	• Short replicative cycle; variable host • Cytopathic • Infect epithelial cells primarily • Latent infection in sensory ganglia
Epstein–Barr virus	• Narrow host range • Replicate in lymphoblastoid tissue • Cause latent infection
Cytomegalovirus	• Replicate slowly • Narrow host range • Cell-associated • Cause cytomegaly

HHV-8	• Replicate slowly • Inactivate tumour suppressor gene • Protein synthesised by HHV-8 known as latency-associated nuclear antigen (LANA) • Inactivate tumour suppressor proteins

HERPES SIMPLEX VIRUS

Clinical Case 1

A 25-year-old woman presented with complaints of genital blisters and fever for last seven days and headache and vomiting for the past two days. On examination, there was neck rigidity. The CSF following lumbar puncture was clear, with a cell count of 100 cells/mm^3 with predominantly lymphocytes, normal glucose and the proteins slightly elevated. PCR test was positive for herpesvirus type 2 *DNA.*

The patient was started on treatment with acyclovir, to which she responded. She was advised to report this episode to her doctor at any future pregnancy.

The herpes simplex virus (HSV) occurs naturally only in humans. The virus is of two types. HSV type 1 (human herpesvirus type 1 or **HHV type 1**) is usually isolated from lesions in and around the mouth and is transmitted by direct contact or droplet spread from cases or carriers. HSV type 2 (**HHV type 2**) is responsible for genital herpes infections and is commonly transmitted venereally. Intracerebral inoculation in rabbits and mice leads to encephalitis, and corneal scarification produces keratoconjunctivitis in rabbits.

The virus grows in a variety of primary and continuous cell cultures like Vero (Fig. 43.2), monkey or rabbit kidney, human amnion, HeLa, producing cytopathic changes, well-defined foci with heaped-up cells and syncytial or giant cell formation. On chick embryo CAM, small (< 0.5 mm), white, shiny and non-necrotic pocks are produced. The two types of virus cross-react serologically.

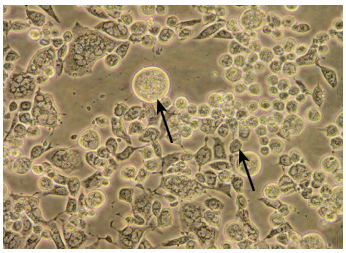


Fig. 43.2 Vero cell line infected with herpes simplex virus: note rounded cells and giant cell formation

Pathogenesis

Herpes simplex is one of the most common viral infections in humans, with about 60–90 per cent of adults showing detectable antibody. **Primary infection** is usually acquired in early childhood, between two and five years of age. Humans are the only natural hosts and the sources of infection are saliva, skin lesions or respiratory secretions. **Asymptomatic carriers** form the more important sources of infection, especially in genital infection with type 2 strains.

Route of infection

Transmission occurs by close contact and may be venereal in genital herpes. The virus enters through defects in the skin or mucous membranes and multiplies locally, with cell-to-cell spread. The virus enters cutaneous nerve fibres and is transported intra-axonally to the ganglia, where it replicates. The virus remains **latent in the ganglia,** particularly of the trigeminal (HSV type 1) and sacral (HSV type 2) nerves, to be reactivated periodically in some individuals, causing recurrent oral and genital lesions.

Antibodies may not prevent recurrences but can reduce the severity of the clinical disease. Herpesvirus diseases are more frequent and severe in HIV-infected and other immunodeficient subjects.

The typical herpes lesions are thin-walled, umbilicated vesicles, the roofs of which break down, leaving tiny superficial ulcers. They heal without scarring. In general, primary infections, though self-limited, are more severe and widespread and associated with systemic manifestations. **Recurrent infections** are more localised.

As a general rule, HSV-1 produces ‘above the waist’ and HSV-2 ‘below the waist’ lesions. However, the

rule is not absolute. HSV-2 infection confers some protection against HSV-1 but not vice versa.

Clinical Features

The clinical manifestations and course depend on the site of infection, age and immune status of the host and the antigenic type of the virus.

Cutaneous infections The most common site is the face—on the cheeks, chin, around the mouth or on the forehead. The typical lesion is the '**fever blister**' or **herpes febrilis**, caused by viral reactivation in febrile patients (Fig. 43.3). In some sensitive persons, very minor stimuli, like the common cold, exposure to sun or even mental strain or menses, may bring on such reactivation.

Fig. 43.3 Fever blister

- **Herpetic whitlow** is a localised infection generally seen on the fingertips of health care workers, doctors and nurses.
- **Eczema herpeticum**, on the other hand, is a disseminated eruption caused by herpes infection in children with eczema. A clinically indistinguishable picture is also produced by vaccinia infection; these symptoms are designated **Kaposi's varicelliform eruption** for both conditions.
- **Erythema multiforme** is a hypersensitive reaction seen in HSV infection. It has typical target lesions on the skin.

Mucosal infections The buccal mucosa is the most commonly affected site. Gingivostomatitis and pharyngitis are the most frequent conditions in primary infection and recurrent herpes labialis in recurrent infection (Fig. 43.4). The vesicles may ulcerate and become secondarily infected.

Fig. 43.4 Herpes simplex lesions on the lips (*Source:* Public Health Image Library, ID 12616 / Robert E. Sumpter / CDC)

Ophthalmic infections HSV infection is the most common cause of **corneal blindness** in some developed countries. Acute **keratoconjunctivitis** may occur by itself or by extension from facial herpes. **Follicular conjunctivitis** with vesicle formation on the lids is another manifestation. The cornea may be involved, with typical branching **dendritic ulcers**. Steroids are contraindicated as they lead to deep stromal involvement and healing may be delayed with scarring and corneal blindness. Chorioretinitis and acute necrotising retinitis are uncommon but serious manifestations.

Infections of the nervous system HSV encephalitis, though rare, is the most common sporadic acute viral encephalitis in most parts of the world. HSV **encephalitis** has an acute onset, with fever and focal neurological symptoms. The demonstration of HSV DNA in CSF by PCR is a very sensitive test in the acute stage (*Clinical case* 1). HSV **meningitis** is a self-limiting disease, usually resolving in about a week without sequelae. The CSF shows lymphocytic pleocytosis and may yield the virus in culture. HSV can cause sacral autonomic dysfunction and also rarely, transverse myelitis or the **Guillain–Barré syndrome**. HSV has been implicated in the causation of Bell's palsy.

Visceral infections HSV **esophagitis** may cause dysphagia, substernal pain and weight loss. It may involve the respiratory tract, causing **tracheobronchitis** and pneumonitis. HSV is an uncommon cause of **hepatitis**. **Disseminated HSV** infection may occur in patients with immunodeficiency, malnutrition or burns.

Genital infections In men, the lesions occur mainly on the penis or in the

urethra, causing **urethritis.** In women, the cervix, vagina, vulva and perineum are affected. When only the cervix is involved, the infection may be asymptomatic. The primary infection is usually more serious, with systemic features like fever and malaise. Recurrent episodes are milder. The vesiculo-ulcerative lesions may be very painful. Rectal and perineal lesions occur in homosexuals. Both types of HSV may cause genital lesions, predominantly, HSV-2.

There have been several reports of an association between HSV-2 and carcinoma of the cervix uteri but a causal relationship has not been established.

Congenital infections Transplacental infection with HSV-1 or 2 can lead to congenital malformations but this is rare. Infection may occur during birth, particularly if the mother has genital lesions due to HSV-2. In such cases, caesarean section may prevent infection. Postnatal infection is more commonly due to HSV-1. **Neonatal herpes** may be confined to the eyes, mouth, skin or it may affect multiple organs, with or without encephalitis. The mortality rate is very high and survivors may have neurological impairment. Prenatal screening for antibodies in mothers using the TORCH (**T**oxoplasma, **R**ubella, **C**ytomegalo and **H**erpes) panel can detect either IgM or IgG, denoting recent or past infection in the mother, thus predicting congenital anomalies or neonatal infection.

Laboratory Diagnosis

1. **Specimens** Vesicle fluid, skin swabs, saliva, corneal scrapings, CSF or brain biopsy on autopsy are used for diagnosis.

The diagnosis of herpesvirus infection may be made by microscopy, antigen or DNA detection, virus isolation or serology.

2. **Microscopy** The **Tzanck smear** is a rapid, fairly sensitive and inexpensive diagnostic method. Smears are prepared from the lesions, preferably from the base of vesicles and stained with 1% aqueous solution of toluidine blue 'O' for 15 seconds. Multinucleated giant cells with faceted nuclei and homogeneously stained **'ground glass'** chromatin (**Tzanck cells**) constitute a positive smear (Fig. 43.5).

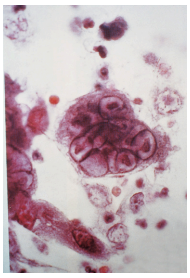


Fig. 43.5 Herpesvirus in Tzanck smear

- **Giemsa-stained smears** show intranuclear type A inclusion bodies.
- **Electron microscopy** can demonstrate the virus particle.
- **Fluorescent antibody technique** demonstrates the herpesvirus antigen in smears or sections from lesions.

3. **Virus isolation** While primary human embryonic kidney, human amnion and several other cells can be used to cultivate the virus, human diploid fibroblasts are preferred. Synticia formation may appear as early as 24–48 hours after infection. However, cultures should be observed for two weeks before being declared negative. Intranuclear inclusion bodies are seen in the infected cells (Fig. 43.5).

Drug susceptibility too can be tested in cell cultures.

> Differentiation between HSV types 1 and 2 may be made by a variety of serological techniques, by nucleic acid hybridisation or by restriction endonuclease cleavage and electrophoretic analysis of viral DNA or viral proteins.

4. **Serology** Serological methods are useful in the diagnosis of primary infections. Antibodies develop within a few days of infection and a rise in titre of antibodies may be demonstrated by ELISA, neutralisation or complement fixation tests. In recurrent or re-infection herpes, there may be little change in the antibody titre.

5. **PCR** For the detection and genotyping of herpes simplex virus (HSV) in clinical specimens, real-time PCR is the method widely used in both research and clinical specimens. It is safer and more sensitive than isolation and immunofluorescence.

Treatment

Acyclovir and vidarabine are effective for the management of deep and systemic infections. Early treatment with intravenous acyclovir has improved the outcome of encephalitis. Oral and topical use may help in less serious conditions. Valaciclovir and famciclovir are more effective oral agents. When resistance to these drugs develops, drugs like foscarnet, which are independent of viral thymidine kinase action, may be useful.

VARICELLA-ZOSTER VIRUS (VZV)

Clinical Case 2

A 50-year-old, HIV-positive man developed painful vesicular eruptions on the right side of his spine in the lumbar region. Scrapings from the base of the lesions were positive for the VZ antigen by the direct fluorescent antibody test. A culture from the fluid was inoculated on cell lines and the PCR test was positive for varicella-zoster virus. The patient was treated with acyclovir. On further enquiry, the patient revealed to the doctor that he had had chickenpox in childhood.

Chickenpox follows primary infection in a non-immune individual, while herpes zoster is a reactivation of the latent virus when immunity has fallen to ineffective levels. Contact with either zoster or chickenpox may lead only to chickenpox but not zoster.

VZV is similar to the herpes simplex virus in its morphology. It does not grow in experimental animals or chick embryos. It can be grown in cultures of human fibroblasts, human amnion or HeLa cells. The cytopathic effects are similar to, but less marked than those produced by the herpes simplex virus. In cultures, the virus remains cell-associated and does not appear free in the medium. By using highly specific antisera, it is possible to distinguish between herpes virus types 1, 2 and varicella-zoster viruses. Only one antigenic type of VZV is known.

Varicella (Chickenpox)

Chickenpox is a common childhood infection. The disease may, however, occur at any age. Adult chickenpox, which is more serious, is rather common in some tropical areas.

The source of infection is a chickenpox or herpes zoster patient. Infectivity is maximum during the initial stages of the disease, when the virus is present abundantly in the upper respiratory tract. **The buccal**

lesions which appear in the early stage of the disease and the vesicular fluid are rich in virus content. Infectivity wanes as the disease progresses and the scabs are virtually non-infectious. There are no animal reservoirs of varicella.

Pathogenesis

The portal of entry of the virus is the respiratory tract or conjunctiva. After an incubation period of about two weeks (7–23 days), the lesions begin to appear. The patient is considered to be infectious two days before and five days after the onset of the lesions.

In children Buccal lesions may not be noticed. The rash usually appears on the trunk. **The evolution of the rash is so rapid that the various stages—macule, papule, vesicle, pustule and scab—cannot be readily followed in individual lesions.** The rash is **centripetal** in distribution, affecting mainly the trunk and sparing the distal parts of the limbs. It is very superficial without involving the deeper layers of the skin, resembling dew-drops lying on the skin. The rash appears in crops during the first three or four days of the disease, so that lesions of varying age can be noticed on the same patient. The lesions mature very quickly, beginning to crust within 48 hours.

In adults When varicella occurs in adults, systemic symptoms may be severe, the rash very profuse and the entire disease much more intense than in children. The rash may become hemorrhagic and occasionally, bullous lesions appear (Fig. 43.6a). Pitted scars on the skin may remain after recovery. Varicella pneumonia is more common in adults, and often fatal in the elderly. Other complications like myocarditis, nephritis, acute cerebellar ataxia, meningitis and encephalitis may ensue. Secondary bacterial infections, usually due to staphylococci or streptococci, may occur. **Reye's syndrome** may follow varicella in some cases with a history of administration of salicylates. One attack confers lasting immunity. Postherpetic neuritis or **shingles** can occur in some individuals, years after the initial infection. It affects a single sensory nerve ganglion, usually in the trunk over one dermatome (Fig. 43.6b). The rashes appear as painful blisters on one side. This is thought to be the reactivation of a latent infection but the exact mechanism of reactivation is not known.

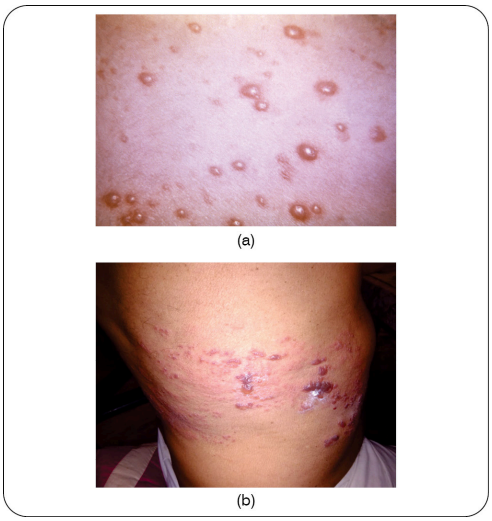


Fig. 43.6 (a) Generalised herpes zoster outbreak due to the varicella-zoster virus (VZV) and (b) Herpes zoster (shingles) affecting a dermatome on the trunk of the patient (*Source:* (a) Public Health Image Library, ID 5407 / Dr. K.L. Hermann / CDC and (b) Dr. Reba / Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Chickenpox in pregnancy Chickenpox in pregnancy can be dangerous for both the mother and the baby. The disease tends to be more severe in pregnant women. If maternal varicella occurs during the first half of pregnancy, the fetal infection is usually asymptomatic. Some infants may develop **fetal varicella syndrome**, manifesting as cicatrising skin lesions, hypoplasia of the limbs, chorioretinitis and CNS defects. Some babies may not exhibit any defects, but may carry latent VZV infection.

Neonatal varicella When maternal varicella occurs near delivery, babies may develop neonatal varicella within two weeks of birth. If the mother's rash begins a week or more before delivery, she will develop antibodies which will be passed, along with the virus, to the fetus transplacentally. Such a baby, though infected, usually escapes clinical disease. If the mother develops chickenpox shortly before (less than a week) or within two days of delivery, the baby receives from the mother only the virus and not the antibody, and so it develops neonatal varicella. This is usually a serious disseminated disease, with a high risk of pneumonia and encephalitis. Such babies are given VZV antiserum and chemotherapy immediately after birth.

Laboratory Diagnosis

Diagnosis is usually clinical.

1. **Microscopy** Multinucleated giant cells and type A intranuclear inclusion bodies may be seen in smears prepared by scraping the base of the early vesicles (Tzanck smears) and stained with toluidine blue, Giemsa or Papanicolaou stain. Electron microscopy of the vesicle fluid may demonstrate the virus with typical herpes morphology.

2. **Virus isolation** This may be attempted from the buccal or cutaneous lesions in the early stages by inoculating human amnion, human fibroblast, HeLa or Vero cells.

3. **Serology** The virus antigen can be detected in scrapings from skin lesions by immunofluorescence, and in vesicle fluid by counterimmunoelectrophoresis with zoster immune serum. The ELISA and PCR techniques are also in use.

Prophylaxis

Vaccine A lyophilised form of the vaccine is now available, which can be stored between 2°C and 8°C. All children should routinely receive the first dose of varicella vaccine (live-attenuated **Oka strain of VZV**) at 12 to 15 months of age. The second dose of the vaccine is recommended at 4 to 6 years of age. It is safe and effective. Occasionally, children may develop a few vesicles which resolve quickly. **It is not considered safe in pregnancy**.

Passive protection **Varicella-zoster immunoglobulin (VZIG)** prepared from the plasma of patients convalescing from herpes zoster provides passive protection in immunocompromised children exposed to infection, but its availability is limited. It is not useful in treatment.

Infection control measures As the infection spreads by direct contact, the affected person is isolated and barrier nursing is done.

Treatment

Specific treatment is indicated mainly in immunodeficient and elderly subjects and those with complications such as varicella pneumonia, encephalitis and disseminated zoster. **Acyclovir and famciclovir** are effective. Corticosteroids are contraindicated in varicella as they enhance the risk of pneumonia and disseminated disease.

HERPES ZOSTER (SHINGLES, ZONA)

While varicella is typically a disease of childhood, herpes zoster (from *herpein*, meaning to creep and *zoster*, meaning girdle) is one of old age, being common after the age of fifty years. The disease may, however, occur at any age and zoster has been reported, very rarely, even in the newborn.

Herpes zoster usually occurs in persons who have had chickenpox several years earlier. The virus, which remains latent in the sensory ganglia, may leak out at times, but is usually held in check by the residual immunity. Years after the initial infection, when the immunity has waned, the virus may be reactivated, and triggered by some precipitating stimulus. Then, it travels along the sensory nerve to produce zoster lesions on the area of the skin or mucosa supplied by it. This reactivation is associated with inflammation of the nerve, which accounts for the neuritic pain that often precedes the skin lesions.

Pathogenicity

The rash is typically unilateral and confined to the area supplied by a single sensory ganglion (*Clinical case 2*). The most common sites are the areas innervated by spinal cord segments D3 to L2 and the trigeminal nerve, particularly, its ophthalmic branch. The lesions are identical to varicella lesions, except for their limited distribution. The rash heals in about two weeks. Pain and paresthesia at the affected area may persist for weeks or months. Other complications are lower motor neuron paralysis which sometimes ensues, meningoencephalitis and generalised zoster, where the lesions are scattered widely, perhaps due to hematogenous dissemination of the virus. **Herpes zoster ophthalmicus** is a common and troublesome presentation. The **Ramsay Hunt syndrome** is a rare form of zoster affecting the facial nerve, with an eruption on the tympanic membrane and the external auditory canal, and often, facial palsy. Chronic or recurrent zoster is often seen in the HIV-infected.

Laboratory Diagnosis

Diagnosis is easily made clinically. Laboratory diagnosis and treatment are as for chickenpox. Herpes zoster represents a mode of evolutionary adaptation by the VZ virus which is an obligate human parasite**. The ability of the virus to remain latent and reappear as zoster years later confers**

on it a great survival advantage.

CYTOMEGALOVIRUSES

Clinical Case 3

A 25-year-old man diagnosed with HIV and AIDS presented with a complaint of diminishing vision, which had been slowly progressive for the previous three months. His CD4 counts at the time of presentation were <100 cells/mm^3. On fundus examination, retinal infiltrates which were fluffy in nature were found, along with multiple hemorrhages. Serological tests were positive for CMV IgG antibodies and PCR on blood was positive for CMV DNA. The patient was diagnosed with CMV retinitis and treatment with ganciclovir was started. Further investigation revealed that the patient had not been taking his anti-HIV treatment regularly; so he was advised to do so again. Subsequently, there was no further deterioration in the patient's condition.

Cytomegaloviruses (CMV), formerly known as **salivary gland viruses**, are a group of ubiquitous herpesviruses of humans and animals. They are characterised by the enlargement of infected cells and prominent intranuclear inclusions. Like other herpesviruses, they lead to prolonged latency in infected hosts. In the neonate and the immunodeficient, they cause severe disseminated disease, while in normal children and adults, the infection is usually asymptomatic or self-limited.

CMV are the largest viruses in the herpesvirus family, with sizes ranging from 150 to 200 nm. The virus exhibits strict host specificity and infection —both in vivo and in vitro—can be established only in the homologous species. Cytomegaloviruses have been identified in human beings, monkeys, guinea pigs and some other species. Human cytomegaloviruses can be grown in human fibroblast cultures. Epithelial cell cultures are not susceptible though epithelial cells are affected in vivo. Cultures have to be incubated for prolonged periods, up to 50 days, as the cytopathic effects are slow in appearance.

Human CMV is unrelated antigenically to other herpesviruses, and even to CMV of other species, except for simian CMV with which some antigenic cross-reaction exists. Minor genetic and antigenic differences exist among human CMV isolates but they are of no clinical significance.

Clinical Features

Cytomegalovirus disease is rare but infection with the virus is extremely

common. As with herpes simplex, the large majority of infections are inapparent, leading to prolonged latency, with occasional reactivation. Clinical disease may be caused by either intrauterine or postnatal infections.

Congenital infections Intrauterine infection leads to fetal death or cytomegalic inclusion disease of the newborn, which is often fatal. This is a generalised infection associated with hepatosplenomegaly, jaundice, thrombocytopenic purpura and hemolytic anemia. The cytomegalic inclusion disease is probably the most important cause of microcephaly. Other manifestations include chorioretinitis and cerebral calcification resembling congenital toxoplasmosis. Survivors may show mental retardation.

Infection in infants Cytomegalic inclusion disease is seen almost exclusively in infants born to mothers who develop primary CMV infection during pregnancy. Infants of mothers who have CMV reactivation during pregnancy tend to have a chronic subclinical infection. Perinatal infection may be acquired from the infected mother through genital secretions or breast milk.

Infection in children Primary infections in older children and adults are usually asymptomatic. However, a heterophile, antibody-negative, infectious mononucleosis may be seen. This is more common following transfusion of CMV-infected blood (**post-transfusion mononucleosis**).

In the immunocompromised host such as an AIDS patient, CMV can cause severe and even fatal infections (*Clinical case* 3). This occurs in transplant recipients, cancer patients on chemotherapy and, more particularly, in the HIV-infected. CMV is an important pathogen in AIDS. In AIDS patients, the already weakened immune response is further damaged by the non-specific CMI-inhibiting effect of CMV. One of the glycoproteins on the surface of CMV acts as a receptor for the *Fc* portion of immunoglobulin molecules. This leads to the masking of the virus by the attachment of irrelevant immunoglobulin molecules, thereby preventing access to specific anti-CMV antibody.

Epidemiology

CMV spreads slowly and probably requires close contact for transmission. It may spread through salivary or other secretions or by sexual contact.

Another method of transmission is by blood transfusion or organ transplants. The virus has been detected in saliva, urine, cervical secretions, semen, blood and milk. Congenitally infected infants have viruria for up to 4–5 years. They are highly infectious in early infancy. About 1 per cent of neonates in the USA are infected with CMV. In the developing countries, the rate may be much higher. Up to 80 per cent of adults show CMV antibodies, indicating the high prevalence of infection. Once infected, the person carries the virus for life.

Laboratory Diagnosis

1. **Specimen** The virus can be isolated from urine, saliva, semen and cervical secretions.

2. **Isolation** Diagnosis may be established by recovery of the virus from urine, saliva or other body fluids by inoculating human fibroblast cultures. Growth in cultures can be detected early by using shell vial cultures and by staining the cells with fluorescent-tagged antibody to early antigens of CMV.

A simpler but less reliable technique is the demonstration of cytomegalic cells in centrifuged deposits from urine or saliva in which inclusion bodies are seen, the appearance of which is described as 'owl's eye'(Fig. 43.7).

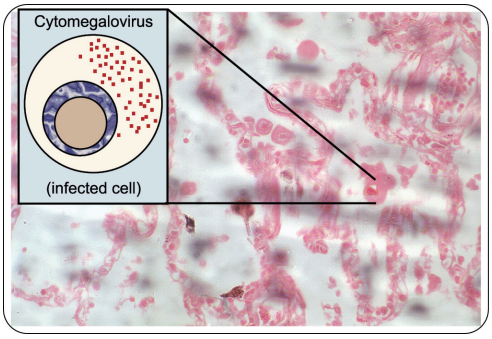


Fig. 43.7 H and E stained lung tissue showing 'owl's eye'-like inclusion bodies of cytomegalovirus ×1,000

3. **Serology** Demonstration of antibody is useful in the diagnosis of primary infection but not in reactivation. Serological techniques in use include CF, IHA, IF and ELISA. Antibody detection may be necessary for screening blood or organ donors. IgM antibody detection helps in the diagnosis of congenital infections.

IgG avidity assay
This test is done to differentiate recent from recurrent infections. The test detects binding affinity or avidity of IgG antibodies. Increase of IgG with high avidity occurs after recurrent infection. In primary infection, high levels of low avidity IgG are detected. Recent infection poses a greater risk in utero to the fetus. Hence detection of low avidity IgG along with IgM, reflects risk to the fetus.

Prevention and Treatment

Prevention is indicated only in high-risk cases such as organ transplant patients, immunodeficient persons and in premature infants. Screening of blood and organ donors and administration of CMV immunoglobulins have been employed in prevention. Acyclovir is useful in prophylaxis but not in treatment. Ganciclovir and foscarnet have been found to be effective and are used for the treatment of CMV disease in patients with AIDS.

No vaccine is available. Experimentally, live attenuated vaccines (Towne 125 and AD 169 strains) and a purified CMV polypeptide vaccine have been found to be immunogenic but not effective in protecting immunodeficient subjects from CMV infection.

EPSTEIN–BARR VIRUS

Clinical Case 4

A 16-year-old girl presented with a complaint of malaise and loss of appetite over the previous month. She also had a sore throat and fever of 40°C with spikes in temperature seen especially in the afternoon, for the previous five days. On examination, cervical lymphadenopathy and mild hepatosplenomegaly were detected. In the complete blood counts, she was found to have absolute mononuclear lymphocytosis, with most of the lymphocytes being atypical. Her serum tested positive for the Paul–Bunnell test. Her IgM was also positive for EBNA. The patient improved on supportive care.

In 1958, Burkitt described an unusual lymphoma among children in certain parts of Africa and suggested, on epidemiological grounds, that the tumour may be caused by a mosquito-borne virus. This led to several attempts at isolating viruses from such tumours.

A number of different viruses, apparently ‘passenger viruses’, were isolated from cultured lymphoma cells. One virus observed in the cultured lymphoma cells by Epstein, Barr and Achong in 1964 was a new type of herpesvirus, named the EB virus, specifically affecting cells of the B lymphocyte lineage. Only human and some subhuman primate B cells have receptors (CD 21 molecules) for the virus. EBV-infected B cells are

transformed so they become capable of continuous growth in vitro.

Epidemiology

The Epstein–Barr (EB) virus is ubiquitous in all human populations. As with other herpesviruses, infection with the EB virus leads to latency, periodic reactivation and lifelong persistence. EB virus antibodies are present in about 95 per cent of adults. In the overcrowded developing world, EB virus infection occurs in infancy and childhood, when it is usually asymptomatic. In affluent countries, primary infection is often delayed until early childhood and adolescence, when it may lead to infectious mononucleosis.

The **source** of the infection is usually the saliva of infected persons who shed the virus in oropharyngeal secretions for months following primary infection and intermittently thereafter. The EB virus is not highly contagious, and droplets and aerosols are not efficient in transmitting infection. Intimate oral contact, as in kissing, appears to be the predominant mode of transmission. This accounts for infectious mononucleosis being called the '**kissing disease**'. Infection may also follow blood or marrow transfusion but these are rare events.

Pathogenicity

The virus enters the pharyngeal epithelial cells through CR 2 (or CD 21) receptors, which are the same as for the C3d component of complement. It multiplies locally, invades the bloodstream and infects B lymphocytes in which two types of changes are produced. In most cases, the virus becomes latent inside the lymphocytes, which become transformed or '**immortalised**' so that they are capable of indefinite growth in vitro.

They are **polyclonally activated** to produce many kinds of immunoglobulins. The heterophile sheep erythrocyte agglutinin seen characteristically in infectious mononucleosis is an example of such polyclonal activation. A second type of effect, shown by a few infected B cells, is **lytic infection** with cell death and release of mature progeny virions.

Mononucleosis represents a polyclonal transformation of infected B lymphocytes. EB virus antigens are expressed on the surface of infected B

cells. The atypical lymphocytes seen in blood smears in infectious mononucleosis are T lymphocytes undergoing blast transformation in response to such neoantigens.

Intermittent reactivation of the latent EB virus leads to clonal proliferation of infected B cells. In immunocompetent subjects, this is kept in check by activated T cells. In the immunodeficient, B cell clones may replicate unchecked, resulting in lymphomas. Hyperendemic malaria prevalent in Africa is believed to be responsible for the immune impairment in children with Burkitt's lymphoma. The frequency of lymphomas seen in many types of immunodeficiencies, most typically in AIDS, may have a similar pathogenesis. Nearly half the lymphomas seen in immunodeficient subjects contain EB virus DNA sequences.

Genetic and environmental factors are said to be important in the nasopharyngeal carcinoma seen in men of Chinese origin. EB virus DNA is regularly found in the tumour cells. These patients have high levels of EB virus antibodies. Genetic influence is best illustrated in the X-linked lymphoproliferative (XLP or Duncan) syndrome associated with extreme susceptibility to EB virus infection.

Clinical Manifestations

EB virus infection may lead to the following clinical conditions:

1. Infectious mononucleosis
2. EBV-associated malignancies:

- Burkitt's lymphoma
- Lymphomas (T cell and B cell) in immunodeficient persons such as those with AIDS and transplant recipients
- Nasopharyngeal carcinoma (seen commonly in persons of Chinese origin)
- Duncan disease, an X-linked lymphoproliferative condition which predisposes a person to EB virus
- Oral hairy leukoplakia (OHL), caused by the Epstein-Barr virus (EBV), commonly seen in HIV infection
- **Hodgkin lymphoma**, in which EBV DNA and RNA are detected in the malignant Hodgkin Reed Sternberg cells

(HRS); the viral nucleic acids present in HRS cells imply that infection of the tumour progenitor cells occurred before the cells proliferated

Infectious Mononucleosis (Glandular Fever)

This is an acute self-limiting illness, usually seen in non-immune young adults following primary infection with the EB virus (Fig. 43.8). The incubation period is 4–8 weeks. The disease is characterised by fever, sore throat, lymphadenopathy and the presence of abnormal lymphocytes in peripheral blood smears (*Clinical case* 4). A mild transient rash may be present. Some patients treated with ampicillin may develop a maculopapular rash due to immune complex reaction to the drug. There is often associated hepatitis, which is usually subclinical and demonstrable only by liver function tests. A number of other complications have been recorded, including hematological, neurological, cardiac and pulmonary conditions and splenic rupture. In most cases, spontaneous resolution of the disease occurs in 2–4 weeks. In some, it may be more prolonged and lead to a state of mental and physical fatigue in convalescence.

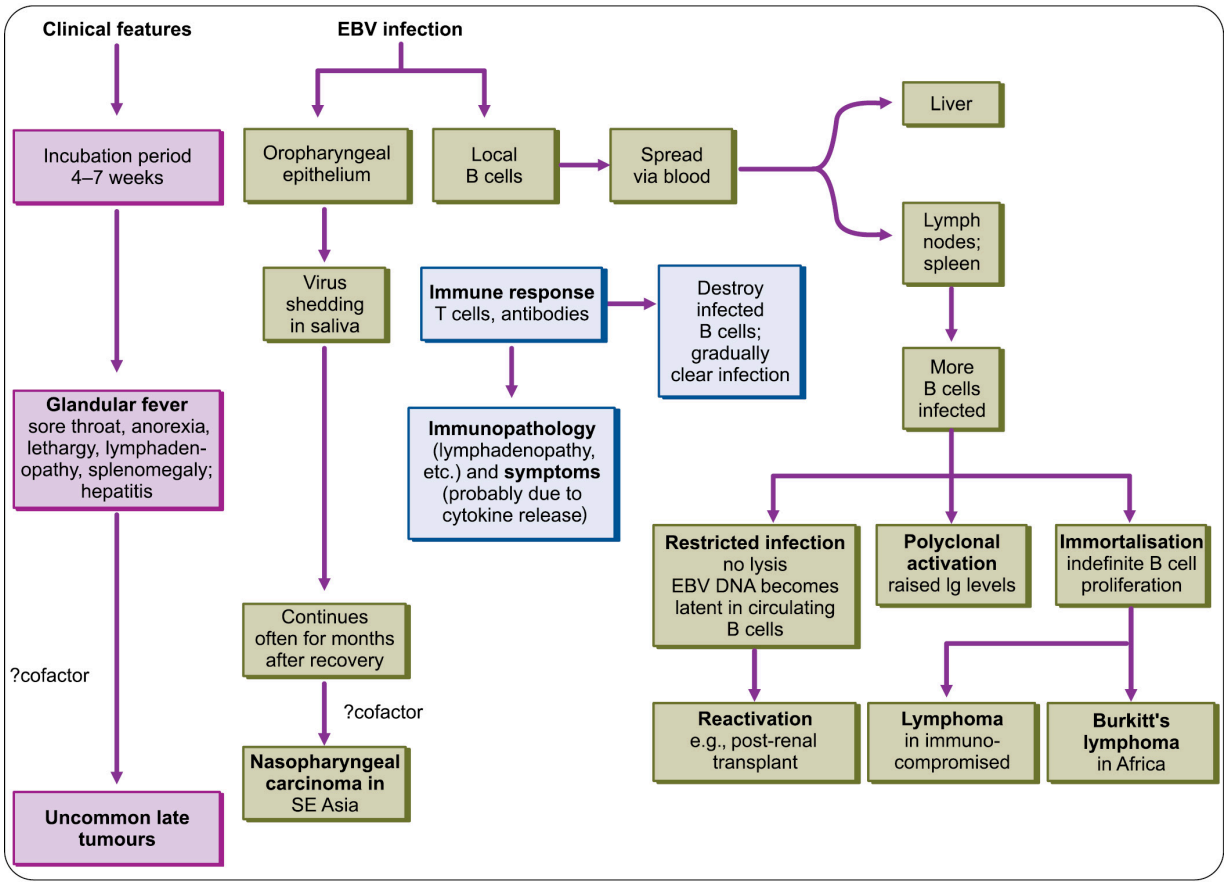

Clinical features
EBV infection
Incubation period 4–7 weeks
Oropharyngeal epithelium
Local B cells
Spread via blood
Liver
Lymph nodes; spleen
Virus shedding in saliva
Immune response T cells, antibodies
Destroy infected B cells; gradually clear infection
More B cells infected
Glandular fever sore throat, anorexia, lethargy, lymphadenopathy, splenomegaly; hepatitis
Immunopathology (lymphadenopathy, etc.) and symptoms (probably due to cytokine release)
Restricted infection no lysis EBV DNA becomes latent in circulating B cells
Polyclonal activation raised Ig levels
Immortalisation indefinite B cell proliferation
Continues often for months after recovery
?cofactor
?cofactor
Reactivation e.g., post-renal transplant
Lymphoma in immuno-compromised
Burkitt's lymphoma in Africa
Nasopharyngeal carcinoma in SE Asia
Uncommon late tumours

Fig. 43.8 Clinical and immunovirological events in Epstein–Barr virus infection

Laboratory Diagnosis

Blood examination during the initial phase may show leucopenia due to a drop in the number of polymorphs. Later, there is prominent leucocytosis, with the appearance of **abnormal mononuclear cells** characterised by deeply basophilic vacuolated cytoplasm and kidney-shaped nuclei showing a lattice of fenestrated chromatin. These atypical mononuclear cells are not virus-infected B lymphocytes but lymphoblasts derived from T cells reactive to the virus infection. The blood picture may sometimes resemble lymphocytic leukemia.

Serology The following serologic tests have replaced the conventional Paul–Bunnell and Monospot tests which depend on heterophile antibodies which are non-specific.

- **Viral capsid antigen (VCA) There are two capsid antigen detection tests to detect IgG and IgM respectively.**
    - **Anti-VCA IgM** appears early in EBV infection and usually disappears within four to six weeks. **Immunofluorescence** and **ELISA** are commonly employed to detect them.
    - **Anti-VCA IgG** appears in the acute phase of EBV infection, peaks at two to four weeks after onset, declines slightly, then persists for the rest of a person's life. It is a reliable indication of primary infection. The IgG anti-VCA antibody persists throughout life and indicates past or recent infection.
- **Early antigen (EA)** Anti-EA IgG appears in the acute phase of illness and generally falls to undetectable levels after three to six months. In many people, detection of antibodies to EA is a sign of active infection. However, 20% of healthy people may have antibodies against EA for years. Antibodies to **early antigens (EA)** are present in high titres in EB-associated lymphomas.
- **EBV nuclear antigen (EBNA)** Antibody to EBNA, determined by the standard immunofluorescent test, is not seen in the acute phase of EBV infection but slowly appears two to four months after the onset of symptoms and persists for the rest of a person's life. **EBNA** is also a useful marker for primary infection. Other EBNA enzyme immunoassays may report false positive results.

Heterophile antibodies (**Paul–Bunnell test**) are detected in infectious mononucleosis. These antibodies agglutinate sheep or horse erythrocytes. However, such antibodies may also occur in serum sickness and hepatitis B and sometimes, even in healthy individuals. These cross-reacting Forssman antibodies can be removed by adsorbing the test serum with guinea pig kidney and ox RBCs prior to testing. It appears that EBV infection alters the cell membrane of infected cells which evoke a non-specific antibody response. These antibodies disappear in six months.

The infectious mononucleosis syndrome can follow infection by other agents such as cytomegalovirus and toxoplasmosis or occur as a reaction to non-infectious stimuli.

HUMAN HERPESVIRUS TYPES 6, 7, 8

A herpesvirus first isolated in 1986 from the peripheral blood of patients with lymphoproliferative disease was called the **human B lymphotropic virus**. It has since been renamed **HHV-6.** This virus is ubiquitous and spreads apparently through saliva in early infancy. Two variants have been recognised: A and B.

Variant B is the cause of a mild but common childhood illness 'exanthem subitum' (known as roseola infantum or 'sixth disease'). In older age groups, it has been associated with infectious mononucleosis syndrome, focal encephalitis and, in the immunodeficient, pneumonia and disseminated disease.

HHV-7 was isolated in 1990 from the peripheral CD4 cells of a healthy person. Like HHV-6, HHV-7 also appears to be widely distributed and transmitted through saliva. It shares with HIV the same CD4 receptor on T cells and could, therefore, contribute to further depletion of CD4 T cells in HIV-infected persons. It has been said to cause some cases of exanthem subitum.

In 1994, DNA sequences presumed to represent a new herpesvirus were identified from tissues of Kaposi's sarcoma from AIDS patients. This was named **HHV-8** and was subsequently identified also in Kaposi's sarcoma in persons not infected with HIV. It is, therefore, referred to sometimes as **Kaposi's sarcoma-associated herpesvirus (KSHV)**. However, a causative relationship is yet to be proved. The incidence is higher in men who have sex with men.

HERPESVIRUS SIMIAE: B VIRUS

This virus was isolated by Sabin and Wright (1934) from the brain of a laboratory worker who developed fatal ascending myelitis after being bitten by an apparently healthy monkey. It came to be known as the 'B' virus, from the initials of the patient. Many similar cases have been reported since then. Herpesvirus simiae infects monkeys in the same manner that herpes simplex infects humans, the infection usually being asymptomatic. The typical lesions produced are vesicles on the buccal mucosa which ulcerate, shedding the virus and infecting contacts. Though most human cases have

followed monkey bites, in some, the infection was acquired through the handling of monkey tissues.

Herpesvirus simiae is similar to herpes simplex virus in its properties. The two are antigenically related but the herpes simplex virus antibody does not protect against herpesvirus simiae infection. A formolised vaccine has been tried experimentally in laboratory workers at risk.

The disease in humans is usually fatal. The rare patients who survive have serious neurological sequelae. The official name for the B virus is **Cercopithecine herpesvirus 1**.

Key points

- All the major herpesviruses are found worldwide. Most adults have antibodies to these viruses, suggesting that exposure is common. However, serious disease is relatively infrequent. Age and immune status affect how infection manifests as disease.
- Herpes simplex virus 1 and 2 (HSV-1 and HSV-2) cause mucocutaneous vesicular and ulcerative lesions. As a general rule, HSV-1 produces 'above the waist' and HSV-2 'below the waist' lesions but this rule is not absolute.
- Infection is transmitted by intimate contact between the infected and the non-infected host: oral or genital (or both). Replication occurs in local epithelial cells until an eruption occurs on the surface as vesicles. Both humoral and cell-mediated immune response are important.
- The infection is prevented by avoiding intimate contact with individuals with lesions. Antiviral drugs (acyclovir) may need to be given in serious infection. There is no vaccine.
- The varicella-zoster virus (VZV) is the cause of chickenpox (varicella), a vesicular rash mainly seen in children that starts at the head and trunk and moves to the extremities. Vesicles may become pustular, crusted and scabbed prior to healing. In adults, VZV infection usually manifests as zoster or 'shingles', characterised by painful vesicular lesions along the distribution of a single nerve.
- Infection is transmitted by the spread of aerosols from an infected individual. Initial infection of epithelial cells of the nasopharynx in a susceptible individual is followed by viral replication, viremia, skin rash and latency in the dorsal root ganglia. Later in life, VZV may emerge to cause zoster. Humoral and cell-mediated immunity are important in determining the outcome of infection.
- A live, attenuated vaccine is available for childhood immunisation. High-titre human immunoglobulin may need to be given for immunocompromised individuals exposed to VZV infection. In individuals who are seriously ill due to VZV infection (encephalitis), antiviral therapy with acyclovir or related drugs may be required.

- Cytomegalovirus (CMV) causes congenital infection of the liver, retina and central nervous system (CNS) with rashes; a mononucleosis syndrome in some older children and adults; it causes a life-threatening disseminated infection in patients with acquired immunodeficiency syndrome and transplant patients which affects the lungs, CNS, liver, retina and gastrointestinal tract.
- The infection is transmitted by intimate contact with secretions (breast milk, saliva and urine) of an infected individual. CMV replicates in the salivary glands and kidney cells, often causing cells to fuse into large, multinucleated giant cells; the virus tends to persist in these tissues, with chronic shedding. As with other herpesviruses, humoral and cell-mediated immunity are important.
- Transmission of infection can be prevented by screening blood and organs before transplants. Specific immunoglobulin against CMV and the antiviral drug ganciclovir have together been found to reduce mortality of CMV pneumonia in bone transplant patients.
- The Epstein–Barr virus (EBV) is known to cause infectious mononucleosis and Burkitt's lymphoma. It has also been implicated in the pathogenesis of nasopharyngeal carcinoma, but not with certainty.
- EBV is transmitted by intimate contact; it grows in B lymphocytes and other lymphatic tissues. Both antibody and cell-mediated immunity are important in determining the outcome of infection.
- In addition to the other diagnostic techniques, infectious mononucleosis can be diagnosed using the Paul–Bunnell test, where heterophile antibodies are sought in the serum of an infected individual which recognise antigens found on sheep and horse RBCs.
- In 1994, a new herpesvirus was identified from tissues of Kaposi's sarcoma from AIDS patients. This was named HHV-8. It has also been identified in Kaposi's sarcoma in persons not infected with HIV. For this reason, it is sometimes referred to as Kaposi's sarcoma-associated herpesvirus (KSHV).
- Herpes simian B virus causes cold sores in monkeys and severe CNS disease in humans. It is a Risk Group 4 pathogen. The unexpected appearance of herpes-like cytopathic effects in primary monkey kidney cells should be investigated with abundant caution in a biological safety cabinet.

Questions

Essays

1. Classify herpesviruses. Mention their general characteristics. Add a brief note on herpes simplex virus.
2. Describe the pathogenesis and laboratory diagnosis of herpes simplex virus infections.
3. Describe the pathogenesis and laboratory diagnosis of the varicella virus.
4. Describe the pathogenesis and laboratory diagnosis of herpes zoster.
5. Describe the pathogenesis and laboratory diagnosis of cytomegalovirus.
6. Describe the pathogenesis and laboratory diagnosis of the Epstein–Barr virus.

Short Answers

1. Diseases caused by HHV-6, 7, 8
2. Infectious mononucleosis
3. Cancers associated with EBV
4. Congenital CMV infections
5. Paul–Bunnell test
6. Tzank smear

Part IV: Virology

CHAPTER 44

Pox and Other Viruses

Chapter navigation

- Pox
- Papilloma
- Polyoma
- Parvovirus

POXVIRUS

Poxviruses are the largest viruses to infect vertebrates. They are large enough to be seen under the light microscope. This group contains several viruses belonging to the family Poxviridae that infect humans, animals, birds and insects. The family includes three medically important viruses:
i) Smallpox (Variola) virus
ii) Vaccinia virus
iii) Molluscum contagiosum virus

Human Infections

Poxvirus diseases are characterised by skin lesions which may be localised or generalised (Fig. 44.1). The most important of these was smallpox caused by the variola virus. Other poxviruses which can infect humans are vaccinia, cowpox, monkeypox, tanapox, molluscum contagiosum, paravaccinia and orf.

Fig. 44.1 Characteristic skin lesions of smallpox in a child (photograph taken prior to global eradication) (*Source:* Public Health Image Library, ID 12783 / World Health Organization (WHO) / Brian W.J. Mahy, BSc, MA, PhD, ScD, DSc / CDC)

Variola and Vaccinia

The variola virus is the causative agent of smallpox. For thousands of years, smallpox raged as a scourge of humans, causing death and disfigurement. Naturally occurring smallpox came to an end in 1977. On 8 May 1980, the WHO formally announced the **global eradication of smallpox**.

The virus responsible for classical smallpox was called **variola major** (Fig. 44.2), a large, brick-shaped virus. A milder form is caused by **variola minor** (alastrim). Variola major and minor were antigenically identical but they differed in certain biological characteristics.

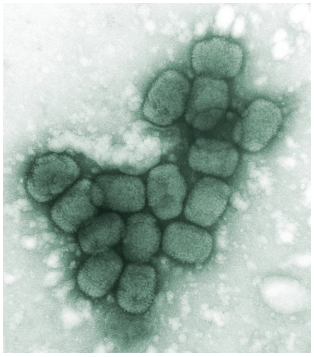


Fig. 44.2 Transmission electron microscopic (TEM) image of a cluster of smallpox viruses, which had been processed using negative stain technique (*Source:* Public Health Image Library, ID 10144 / Dr. Fred Murphy / CDC)

Vaccinia and the smallpox vaccine The vaccinia virus was used as the smallpox vaccine. Jenner originally used the cowpox virus for vaccination against smallpox. The vaccinia virus is unique in that it is an 'artificial virus' and does not occur in nature as such. The vaccinia genome can accommodate about 25,000 foreign base pairs, sufficient for introducing

several genes. Many genes have been inserted into it including those coding for the antigens of the hepatitis B virus, HIV and rabies. However, the vaccinia virus is not suitable as a vector for human use due to its pathogenic effects.

Morphology

The virion is brick-shaped (Fig. 44.3). In vertical section, it consists of a double-layered membrane which surrounds a biconcave 'nucleoid' containing the DNA core. On either side of the nucleoid, is a lens-shaped structure called the lateral body. The virion measures about 300 × 200 × 100 nm and, therefore, can be seen under the light microscope.

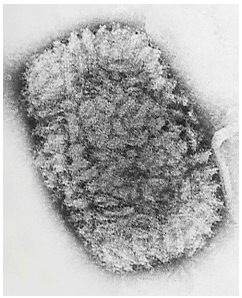


Fig. 44.3 Transmission electron microscopic (TEM) image depicting a single, barrel-shaped, vaccinia virus particle; it is used therapeutically in the form of a vaccine (*Source:* Public Health Image Library, ID 1877 / Dr. Fred Murphy; Sylvia Whitfield / CDC)

Physical and Chemical Properties

Poxviruses are stable and, if protected from sunlight, may remain viable for months at room temperature. In the cold or when freeze-dried, they survive for years. They are susceptible to ultraviolet light, other irradiations, formalin and oxidising disinfectants. They are resistant to 50% glycerol and 1% phenol.

Antigenic Structure

All poxviruses share a common nucleoprotein (NP) antigen.

Cultivation and Host Range

The variola and vaccinia viruses can be differentiated by their growth characteristics and host range.

Chick embryo Both viruses grow on the chorioallantoic membrane of 11–

13-day-old chick embryo, producing pocks in 48–72 hours. Variola pocks are small, shiny, white, convex, non-necrotic and non-hemorrhagic lesions (Fig. 44.4). Vaccinia pocks are larger, irregular, flat and greyish necrotic lesions, some of which are hemorrhagic.

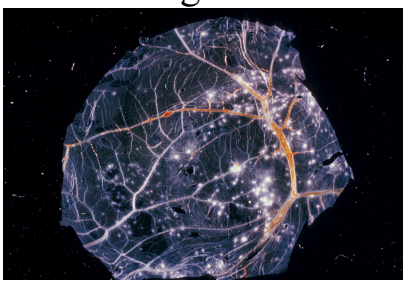


Fig. 44.4 Photograph showing smallpox virus pocks on the chorioallantoic membrane (CAM) of a developing embryonic chick (*Source:* Public Health Image Library, ID 3274 / John Noble / CDC)

Tissue culture Variola and vaccinia viruses can be grown in tissue cultures of monkey kidney, HeLa and chick embryo cells. Cytopathic effects are produced by vaccinia in 24–48 hours and more slowly by variola. Eosinophilic inclusion bodies—**Guarnieri bodies**—can be demonstrated in stained preparations. The inclusion bodies consist of aggregations of virus particles in a matrix.

Animals The vaccinia virus can infect a wide range of animals experimentally. Monkeys, calves, sheep and rabbits can be infected by scarification, leading to vesicular lesions. The variola virus produces similar lesions only in monkeys.

Smallpox

Smallpox has been eradicated. The last natural case of variola major detected was Saiban Bibi, a Bangladeshi woman found with smallpox on the Karimganj railway platform in Assam on 24 May 1975. The last case of variola minor occurred in Merca, Somalia, in October 1977. The coming generations are unlikely to witness the disease but its disappearance has been too recent for it to be ignored altogether. A brief account of smallpox is, therefore, being presented here.

Briefly, the pathogenesis begins when the virus enters the body by inhalation. After initial multiplication in the local lymphoid tissues, the virus reaches the reticuloendothelial cells, where further multiplication takes place, leading to severe viremia with seeding of the mucosa and skin,

heralding the clinical disease. The incubation period is around 12 days. A single crop of centrifugal exanthems passes through the macular, papular, vesicular and pustular stages, before scabbing and healing by scar formation in 2–4 weeks. The exanthems vary in severity from the hemorrhagic, flat, ordinary or modified form, in descending order.

Though natural smallpox ceased in 1977, a small outbreak of variola major occurred in August 1978, in Birmingham, following the accidental spread of the virus from a laboratory in a medical school. It was promptly identified and controlled. Following this, the WHO

issued a directive for all such laboratory stocks of the virus to be destroyed. The last stocks of the smallpox virus were held under high security in the Centers for Disease Control and Prevention, Atlanta, Georgia (USA) and the Centre for Research on Virology and Biotechnology, Koltsova (Russia). They were to have been destroyed by June 30, 1999. As a measure of protection against the remote danger of smallpox re-emerging or posing a bioterrorism threat, large stocks of smallpox vaccine are maintained by the WHO for rapid deployment, if needed.

Other Poxvirus Diseases

With the elimination of smallpox, it has become important to identify and characterise other orthopoxviruses that can infect human beings and cause diseases resembling smallpox.

Monkeypox This virus was first isolated in 1958 from an outbreak of pox disease in a captive monkey colony in Copenhagen. Monkeys are only incidental hosts, rodents being the natural hosts. The first human case was reported in 1970 from Zaire. Human infection is common in Central and West Africa, with a fatality rate of 5–10 per cent. In 2003, an outbreak occurred in Wisconsin, USA, affecting 11 local persons and many prairie dogs. The source of infection is said to have been an imported African rodent, which infected local human contacts and prairie dogs. Serological studies have shown evidence of widespread natural infection in monkeys in Africa. The virus can be distinguished from variola.

Buffalopox This was identified in cattle in India in 1934 and was considered as an outbreak of vaccinia. Epizootics had occurred in buffaloes and lesions had been observed on the hands of persons in contact with infected animals. Buffalopox still occurs, proving it to be distinct from variola and vaccinia. The smallpox vaccine does not seem to protect persons against occupational buffalopox.

Cowpox and milker's nodes Both these infections are obtained from

cows. Cowpox lesions are seen on the udder and teats of cows and may be transmitted to humans during milking. The lesions in humans usually appear on the hands or fingers and resemble primary vaccinia. Restriction endonuclease maps of vaccinia and cowpox genomes show distinct differences.

Cowpox infection has been observed only in Britain and Europe.

Milker's node (paravaccinia) is a trivial occupational disease that humans contract by milking infected cows.

Orf (contagious pustular dermatitis) Orf is a disease of sheep and goats transmitted to humans by contact. In humans, the disease occurs as a single papulovesicular lesion with a central ulcer, usually on the hand, forearm or face. The virus is unrelated to the variola–vaccinia group and resembles the paravaccinia virus morphologically.

Tanapox This virus was isolated from epidemics of a febrile illness along the Tana river in Kenya in 1957–62. The patients had a single pock-like lesion on the upper part of the body. The virus is active in Africa, particularly in Zaire.

Molluscum contagiosum This disease, seen usually in children and young adults, is characterised by pink or pearly white wart-like nodules on the skin (Fig. 44.5a). Sections of the lesions show large (20–30 mm) eosinophilic hyaline inclusion bodies which displace the nuclei to the margin. These **molluscum bodies** are composed of large numbers of virus particles, embedded in a protein matrix (Fig. 44.5b). Humans are the only susceptible hosts. The virus can't be grown in eggs, tissue cultures or animals. The incidence of molluscum contagiosum as a sexually transmitted disease in young adults is increasing. When it occurs in the genital area, it may become inflamed and ulcerated and may simulate HSV infections.

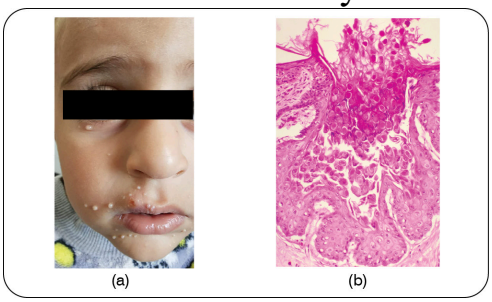


Fig. 44.5 (a) Molluscum contagiosum: pearly white wart-like nodules on the skin and (b) photomicrograph of a skin tissue specimen, revealing the histopathologic changes and

large numbers of virus particles embedded in a protein matrix in a case of molluscum contagiosum (
Source: (a) https://upload.wikimedia.org/wikipedia/commons/thumb/e/e0/Molscum_contagusum and
(b) Public Health Image Library, ID 860 / Dr. Edwin P. Ewing, Jr. / CDC)

Yabapox This is a monkey tumour virus which is related to poxviruses.

PAPILLOMAVIRUS

Papillomavirus family is a large group of DNA viruses, which were formerly part of the Papovaviridae family. They contain five genera which cause infection in humans. They are the alpha, beta, gamma, mupa and nupa papillomaviruses. They are icosahedral, approximately 55 nm in diameter, with double-stranded DNA.

Significant characteristics of the virus are:

- It presents tissue tropism to epithelial cells of the skin and mucous membrane.
- Its host range is restricted to primates.
- It stimulates cell DNA synthesis.
- The viral oncoproteins interact with tumour suppressor proteins of human cells.
- The virus is associated with cervical, anogenital and other human cancers.

Human papillomavirus is the most common sexually transmitted infection. Most sexually active men and women are exposed to the virus at some point in their lifetime. The virus can be spread through oral, vaginal, or anal sex. Sometimes, HPV can be transmitted to an infant during the birth, causing genital or respiratory system infections. It causes genital warts (Fig. 44.6). The incidence of infection is the highest among adolescents and young adults.

Fig. 44.6 Genital wart caused by human papillomavirus (*Source:* Public Health Image Library, ID 4151 / Dr. Wiesner / CDC)

The virus does not have an in vitro assay and hence neutralisation tests commonly used for other viral assays cannot be applied to papillomavirus. They are typed by molecular methods. Around 200 HPV types have been identified. Several types are specific for certain clinical lesions. HPV-16 and 18 are associated with almost 70% of cervical cancers (Table 44.1).

Table 44.1 Human papillomaviruses and the human infections they cause

Clinical lesions	Human papillomavirus type
Plantar warts	1
Skin warts	2, 4, 27, 57
Cutaneous lesions, anogenital condyloma, laryngeal papilloma	3, 10, 28, 49, 60, 76, 78
Genital carcinoma (including cervical carcinoma), high-grade dysplasias, laryngeal and oesophageal carcinomas	16, 18, 30, 31, 35, 39, 45, 51–53, 56, 58, 59, 66, 68, 73, 82

There is **no treatment** available for HPV infection. Salicylates have been used to treat some warts.

Prevention is through the HPV vaccine. Adolescent children between 11 to 12 years of age are vaccinated against HPV. There are two types of vaccines:

- Bivalent vaccine contains HPV types 16 and 18
- Quadrivalent vaccine contains HPV types 6, 11, 16 and 18

Vaccination is contraindicated in pregnant women.

POLYOMAVIRUS

This group contains a single genus. Viruses belonging to this group possess a circular genome of double-stranded DNA within a non-enveloped capsid. Human viruses are JC, BK, KI, WU, HPyV6, HPyV7 and Merkel cell virus. SV40 is found in monkeys, which may pose a problem in using monkey-derived cell lines for human virus culture (it accidentally contaminates early killed and live poliovirus vaccine grown on monkey cells).

Human polyomaviruses are widely distributed in the population.

JC and BK viruses may remain in kidney and lymphoid tissue after primary infection in healthy individuals. They may get reactivated in immunosuppressed conditions, e.g., during renal transplantation, in AIDS patients, etc., causing progressive multifocal leukoencephalopathy (PML).

Ki and Wu polyomaviruses have been associated with respiratory infections in children. Merkel cell carcinoma is a rare neuroendocrine tumour.

PARVOVIRUS

Parvovirus is the smallest DNA virus; it is incapable of replicating without the function of the host cell or a co-infecting helper virus. **Parvovirus B19** is pathogenic to humans, causing an infection known as **erythema infectiosum** (**fifth disease**). The virus has a tropism to progenitor erythroid cells. Fifth disease is a common exanthematous infection in childhood.

The virus is non-enveloped, icosahedral, measures 18–26 nm and has 32 capsomeres.

Replication of the virus occurs in primary erythroid progenitor cells which are permissive for B19. The receptor for the virus is the **P-antigen** expressed on erythrocytes, erythroid progenitor cells and several other cells like endothelial cells, placenta, fetal liver and heart cells.

The virus multiplies in the adult bone marrow and some blood cells.

Human diseases associated with B19p parvovirus are:

- Erythema infectiosum in children, leading to cutaneous rash and arthralgia in adults.
- Transient aplastic crisis, causing severe anemia which is acute.
- Pure red aplasia causing chronic anemia.

- Hydrops fetalis, leading to fetal anemia which may cause fetal death.

Laboratory diagnosis of B19 viral infection can be made by PCR. Viral load assays are done to detect copies of the genome in serum, blood cells and tissue samples.

The detection of IgM antibodies against a B19 viral protein (VP2) in the serum by ELISA is indicative of recent infection. IgG antibodies persist for years.

Treatment is symptomatic as there are no antiviral agents known to be effective.

Key points

- The poxviruses are large, being 250–300 nm by 300–350 nm in size, and just visible under a light microscope. The genus *Orthopoxvirus* includes the viruses causing cowpox, vaccinia and variola.
- Cowpox is an orthopoxvirus of cattle of the type thought to have been used by Jenner as a vaccine against smallpox.
- The vaccinia virus is an orthopoxvirus and is closely related to the variola virus. It was used as a vaccine for the control and final eradication of smallpox.
- The variola virus causes smallpox. It has been eradicated as a naturally occurring pathogen and most of the stocks of the virus have also been destroyed.
- The monkeypox virus is an orthopoxvirus of monkeys, closely resembling the variola virus. It is zoonotic in humans, causing a relatively mild, smallpox-like illness.
- The virus causing molluscum contagiosum is a poxvirus. It causes pink or pearly white warty lesions of the skin in children and young adults. The incidence of this disease as a sexually transmitted disease is increasing; lesions on the genitalia may become secondarily infected by HIV.
- The orf virus is a parapoxvirus of sheep and goats that causes zoonotic infection in humans.
- **Human papillomavirus** is the most common sexually transmitted infection. Most sexually active men and women are exposed to the virus at some point in their lifetime. The virus can be spread through oral, vaginal or anal sex.
- It has tissue tropism to epithelial cells of the skin and mucous membrane.
- The viral oncoproteins interact with tumour suppressor proteins of human cells. They are associated with cervical, anogenital and other human cancers.
- **Parvovirus B19** is pathogenic to humans, causing an infection known as **erythema infectiosum** (**fifth disease**). The virus has a tropism to progenitor erythroid cells. Fifth

disease is a common exanthematous infection in childhood.

Questions

Short Answers

1. Important orthopoxviruses and the diseases caused by them
2. Smallpox—present status
3. Molluscum contagiosum
4. Vaccinia virus
5. Papillomavirus infection in humans
6. Fifth disease

Part IV: Virology

Chapter 45

Adenovirus

Chapter navigation

- Adenovirus
- Adeno-associated virus

INTRODUCTION

Adenoviruses are a group of medium-sized, non-enveloped, double-stranded DNA viruses that share a **common complement-fixing antigen**. They infect humans, animals and birds, showing **strict host specificity.** Adenovirus infections are common worldwide, mostly in children. Several of the infections are asymptomatic. The virus may persist in the host for many months. Adenoviruses cause infections of the respiratory tract and eyes, and less often, of the intestine and urinary tract.

In 1953, Rowe and associates grew surgically removed human adenoid tissue in plasma clot cultures and noticed that the epithelial outgrowths underwent spontaneous degeneration resembling viral cytopathic change. The isolated viruses were subsequently designated as adenoviruses.

ADENOVIRUSES

Clinical Case

During the monsoon, 10 students from a boarding school complained of irritation and redness in the eyes along with mild fever and sore throat. Some of them had severe pain in the eye with excessive watering and corneal ulcers. Conjunctival swabs were positive for adenovirus type 8 IIF. PCR was also positive for adenovirus type 8 in all the students. Supportive therapy was given for corneal ulcers. While the students with conjunctivitis responded in eight days, the corneal ulcers took three weeks to heal. The children were advised precautionary isolation, and further spread was controlled.

Morphology

Adenoviruses are 70–75 nm in size. They have a characteristic morphology. The capsid is composed of 252 capsomers arranged as an icosahedron with

20 triangular facets and 12 vertices (Fig. 45.1a). Of the 252 capsomers, 240 have six neighbours and are called hexons, while the 12 capsomers at the vertices have five neighbours and are called pentons. Thus, the virion has the appearance of a **space vehicle** (Fig. 45.1b).

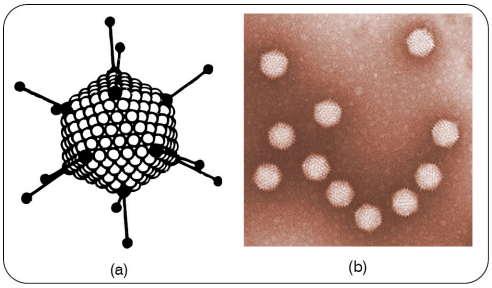


Fig. 45.1 Adenovirus: (a) diagrammatic representation of structure and morphology and (b) microscopic view

Classification

Over 50 serotypes of adenoviruses have been isolated from human sources and are divided into groups A–F (Table 45.1). Most of the recent serotypes were recovered from AIDS patients. They infect only the homologous species, with the exception of oncogenic human adenoviruses (types 12, 18, 31) that cause sarcomas in newborn hamsters.

The group antigens are present mainly on hexons and can be detected by immunofluorescence or ELISA. Type-specific antigens are located on pentons and fibres. Serotypes are identified by the neutralisation test. **Human adenoviruses are classified into six groups** (also called subgroups or subgenera) based on properties such as hemagglutination, fibre length, DNA fragment analysis and oncogenic potential.

Table 45.1 Classification of human adenoviruses

Group (Subgenus)	Serotype (Species)	Hemagglutination		Oncogenic potential	
		Red cells	Pattern	Tumouro-genicity	Transformation of in vivo cultured cells
A	12, 18, 31	Rat	±	High	+
B	3, 7, 11, 14, 16, 21, 34, 35	Monkey	+	Weak	+
C	1, 2, 5, 6	Rat	±	Nil	+

D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–47	Rat	+	Nil	+
E	4	Rat	±	Nil	+
F	40, 41	Rat	±	NK	+

+ = complete hemagglutination; ± = partial hemagglitination and NK = not known.

Resistance

Adenoviruses are relatively stable, remaining viable for about a week at 37°C. They are readily inactivated at 50°C. They resist ether and bile salts.

Pathogenicity

Adenoviruses cause infections of the respiratory tract, eye, bladder and intestine. More than one type of virus may produce the same clinical syndrome, and one type of virus may cause clinically different diseases (Table 45.2).

Table 45.2 Common syndromes associated with adenovirus infection

Syndrome	Principal serotypes
Respiratory disease in children	1, 2, 5, 6
Sore throat, febrile cold, pneumonia	3, 4, 7, 14, 21
ARD in military recruits	4, 7, 21
Follicular (swimming pool) conjunctivitis	3, 7
Epidemic keratoconjunctivitis (shipyard eye)	8, 19, 37
Diarrhea	40, 41
Pneumonia, hepatitis, colitis, etc., in transplant recipients	34, 35

The following syndromes have been recognised to be associated with certain serotypes of adenovirus:

- ***Pharyngitis*** Adenoviruses are the main cause of non-bacterial pharyngitis and tonsillitis, presenting as febrile common cold. **Types 1–7** are commonly responsible.

- ***Pneumonia*** Adenovirus **types 3 and 7** are associated with pneumonia in adults, resembling primary atypical pneumonia. In infants and young children, type 7 may lead to more serious and even fatal pneumonia.
- ***Acute respiratory diseases (ARD)*** This occurs usually as outbreaks in military recruits. **Serotypes 4, 7 and 21** are the agents commonly isolated.
- ***Pharyngoconjunctival fever*** This syndrome of febrile pharyngitis and conjunctivitis seen in the civilian population is usually associated with **serotypes 3, 7 and 14.**
- ***Epidemic keratoconjunctivitis (EKC)*** This is a serious condition which may appear as an epidemic, usually caused by **type 8** and less often, by types **19 and 37** (*Clinical case*).
- ***Acute follicular conjunctivitis*** This is a non-purulent inflammation of the conjunctiva with the enlargement of the submucous lymphoid follicles and of the pre-auricular lymph nodes. **Types 3, 4 and 11** are commonly responsible. Adenoviral and chlamydial conjunctivitis are clinically similar.
- ***Diarrhea*** Adenoviruses can often be isolated from feces but their connection to intestinal disease has not been conclusively established. However, some fastidious adenoviruses can cause diarrheal disease in children (**types 40** and **41**). They have been designated as **enteric type adenoviruses**. They are not grown in a routine cell culture and special techniques of tissue culture (in trypsinised monkey kidney cells or transformed human embryonic kidney cells) have been developed for their cultivation. They can also be identified by stool ELISA.
- ***Acute hemorrhagic cystitis in children and generalised exanthem*** These are two other syndromes that have been reported. Adenoviruses **types 11 and 21** are responsible for the former.
- ***Mesenteric adenitis and intussusception*** Adenoviruses have been isolated from mesenteric lymph nodes of children with such complications.
- ***Transplant recipients*** Adenoviruses cause infection in immunosuppressed individuals. Types 34 and 35 have been

commonly associated with organ recipients.

Laboratory Diagnosis

Diagnosis can be established by:

1. **Tissue culture** Human adenoviruses grow only in tissue cultures of human origin such as human embryonic kidney, HeLa or HEp-2. Cytopathic changes may take several days to develop and consist of cell **rounding and aggregation** into grape-like clusters. **Intranuclear inclusions** may be seen in stained preparations.

Shell vial technique Isolation of the virus from the throat, eye, urine or feces can be hastened by shell vial culture technique, which increases the rate of isolation. The processed specimen is inoculated on to shell vials with monolayers of cell line, and centrifuged for 15 minutes at 4,300 rpm (3,500 ×g). This increases the adsorption of viral particles onto the cells, thus facilitating adherence and penetration into the cells. The time to positivity decreases. Cytopathic effect can be seen by direct fluorescent or immunofluorescent staining.

Other methods of identification are:

- Noting the cytopathic effects
- Complement fixation tests with adenovirus antiserum
- Subgrouping by hemagglutination with rat and monkey erythrocytes
- Typing by neutralisation tests

2. **Serodiagnosis** Rising antibody titre in paired sera is diagnostic. **Examination of a single sample of serum is inconclusive as adenovirus antibodies are so common in the population.**

3. **Electron microscopy** is used for fecal virus detection.

4. **Immunofluorescence** for viral antigen detection in nasopharyngeal and ocular infections is useful.

5. **PCR-based tests** have been used for the detection of viral DNA.

Prophylaxis

Killed and live vaccines have been used for the prevention of ARD with

some success. No vaccine is available for general use.

Gene therapy Adenoviruses appear to have a spare capacity to carry DNA inserts of up to 7 kb and are being investigated as potential vectors in gene therapy.

ADENO-ASSOCIATED VIRUSES (AAV)

Electron microscopy of adenovirus preparations have revealed small icosahedral viral particles, 20–25 nm in diameter. They are unable to replicate independently and are called defective viruses or viruses dependent on helper viruses. They can multiply only in cells simultaneously infected with adenoviruses and are called **adeno-associated viruses** (AAV) or adenosatellite viruses. They have been classified as the genus *Dependovirus* (referring to their dependence on adenoviruses) under the family Parvoviridae. They can be detected by electron microscopy and complement fixation or immunofluorescence with specific antisera. Types 1, 2 and 3 are of human origin and cause natural infection.

Key points

- A bacteriophage is a type of virus that specifically infects bacteria and fungi.
- Bacteriophages exhibit two different types of life cycles:
    - **Lytic** (virulent) cycle, where intracellular multiplication of the phage ends in the lysis of the host bacterium and the release of progeny virions.
    - **Lysogenic** (temperate) cycle, where phage DNA becomes incorporated within the bacterial genome and then replicates synchronously with it, causing no harm to the host cell.
- The lytic cycle replication allows the bacteriophage to proceed through the stages of adsorption (attachment to the host cell), penetration of the nucleic acid into the cell, synthesis of phage components, assembly of phage components and maturation and release of progeny phage particles from the cell.
- Phages play an important role in the transmission of genetic information between bacteria by the process of transduction. They can carry antibiotic resistance genes or virulence genes. Bacteria can be typed by phage typing methods for epidemiological purposes.

Questions

Essay

1. Describe the lytic cycle of bacteriophages.

Short Answers

1. Structure of a bacteriophage (draw and describe)
2. Phage typing (definition)
3. Lysogeny (definition)
4. Uses of bacteriophages

Part IV: Virology

Chapter 46

Picornavirus

Chapter navigation

- Enterovirus
  - Polio
  - Coxsackie A and B
  - ECHO
  - Enterovirus 68-116
- Rhinovirus

INTRODUCTION

The family Picornaviridae comprises a large number of very small (*pico* meaning 'small') RNA viruses. They are non-enveloped viruses, 27–30 nm in size, resistant to ether and other lipid solvents and are divided into two main groups, *Enterovirus* and *Rhinovirus*. These groups, as the name suggests, may be found transiently in the alimentary tract or the nasopharynx respectively. However, they cause a number of varied infections in susceptible individuals.

Classification

Besides the two groups of picornavirus which comprise the entero- and rhinoviruses, several other viruses have been brought into this category due to the similar gastrointestinal and respiratory illness caused by them.

Four genera of picornaviruses are of medical importance:

i) *Enterovirus*, which infects the intestinal tract
ii) *Rhinovirus*, which infects the nasopharynx
iii) *Hepatovirus*
iv) *Parechovirus*

Two other picornavirus genera of veterinary importance are:

- *Aphthovirus*, which causes foot-and-mouth disease in cattle
- *Cardiovirus*, which causes encephalomyocarditis of mice

ENTEROVIRUSES

Most enteroviruses are host-specific, infecting only one or a few related species. There is no common group antigen for enteroviruses, though some of them show antigenic cross-reactions. They are found in the gastrointestinal or nasopharyngeal track, hence the name *Enterovirus* and *Rhinovirus*.

The introduction of tissue culture techniques in diagnostic virology led to the isolation of several cytopathogenic viruses from the feces of sick as well as healthy persons. They were called orphan viruses because they could not be associated with any particular clinical disease. They came to be known by the descriptive term **enteric cytopathogenic human orphan** (**ECHO**) viruses.

Paralytic disease of children has been recognised from very early times. Experimental transmission of the disease to monkeys was demonstrated by Landsteiner and Popper. **Enders, Weller** and **Robbins** (1949) demonstrated that polioviruses could grow in cultures of non-neural cells from human embryos, producing cytopathic effects, a discovery for which they were awarded the **Nobel Prize.** Dalldorf and Sickles (1948) demonstrated the Coxsackie virus from the feces of children with paralytic poliomyelitis. These patients came from the village of Coxsackie in New York, hence the name.

Classification

Enteroviruses of medical importance include:

Poliovirus types	1–3
Coxsackievirus A types	1–24 (types not included: 15, 18 and 23)
Coxsackievirus B types	1–6
Echovirus types	1–34 (types not included: 8, 10, 22, 23, 28 and 34)
Enterovirus types	68–116 (type not included: 72)

Retaining the enterovirus nomenclature, coxsackie A virus now falls into human enterovirus species A (HEV-A) and HEV-C, while coxsackie B virus falls into HEV-B which also includes ECHO virus. Newer enteroviruses after 1969 have been assigned enterovirus (EV) type numbers, starting with 68.

Enterovirus 72, which causes infectious hepatitis (hepatitis type A), has been reclassified as a separate genus ***Hepatovirus***.

Rhinoviruses were considered a separate genus earlier, but are now included in the genus *Enterovirus*. They have 115 antigenic types and are classified into 3 species of human rhinovirus (HRV) A, B and C.

POLIOVIRUS

Clinical Case

A 10-year-old boy from a labour colony presented to the emergency department with a history of mild fever, headache and sore throat for the previous eight days. This was accompanied by vomiting for the previous two days. There was a brief asymptomatic period of two days. On the morning of presentation, he experienced pain in the right lower limbs, followed by weakness, and inability to walk at the time of presentation. His mother gave no history of vaccination during the first five years of the boy's life, and he had missed the mass vaccination campaign. Throat and rectal swabs and cerebrospinal fluid (CSF) were sent for viral studies to a referral laboratory. The throat and rectal swabs showed cytopathic changes in primary monkey kidney cell line which was confirmed as poliovirus type 1 by the neutralisation test. Reverse transcriptase PCR in CSF was positive for poliovirus. The general condition of the child improved on supportive treatment.

Morphology

The virion is a spherical particle, about 27 nm in diameter, composed of 60 subunits, each consisting of four **viral proteins** (VP1–VP4), arranged in icosahedral symmetry. VP1, which faces outside, carries the major antigenic site for combination with type-specific neutralising antibodies. The genome is a **single strand of positive-sense RNA** of 7.4 kb. It can be directly translated by host ribosomes to form a polyprotein which is cleaved into 11 different proteins. The positive-sense genome RNA is infectious.

The virus can be crystallised, and arrays of virus crystals can be seen in the cytoplasm of infected cells (Fig. 46.1 a and b).

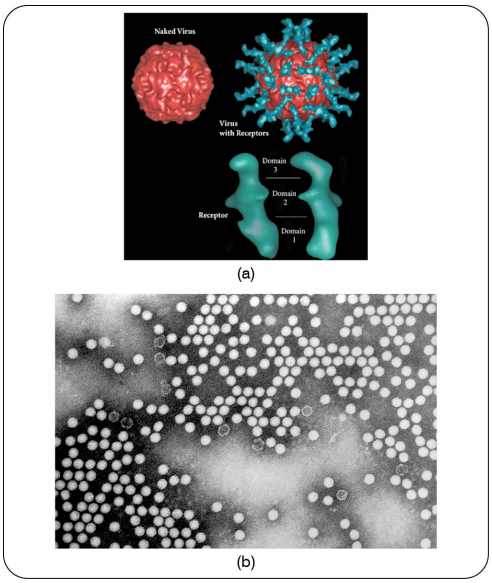


Fig. 46.1 (a) Structure of poliovirus and (b) (TEM) image depicting numerous, round, poliovirus type-1 virions, measuring 20–30 nm in diameter with icosahedral symmetry (*Source:*
(b) Public Health Image Library, ID 235 / Dr. Joseph J. Esposito; F. A. Murphy / CDC)

Antigenic Types

There are three antigenic types:

1. **Type 1** (Brunhilde and Mahoney strains) is the most common and causes most epidemics.
2. **Type 2** (Lansing and MEFI strains) usually causes endemic infections.
3. **Type 3 (**Saukett strains) have caused epidemics. Immunity is type-specific.

Two antigens, C and D, can be recognised based on **complement fixation, ELISA or precipitation tests**.

1. The **C antigen** (**coreless**) also called the **heated or H antigen,** is associated with the 'empty' non-infectious virus. Anti-C antibody does not neutralise virus infectivity and is not protective.
2. The **D antigen** (**dense),** also called the **native or N antigen**, is associated with the whole virion and is type-specific.

The D antigen is converted into the C antigen by heating the virus at 56°C. Anti-D antibody is protective. **Potency of the injectable polio vaccine can be measured in terms of D antigen units.**

Resistance

- Poliovirus is resistant to ether, chloroform, bile, proteolytic enzymes of the intestinal contents and detergents. It is stable at a pH of 3. In feces, it can survive for months at 4°C and for years at –20°C. Depending on conditions like temperature, moisture, pH and the amount of virus, its survival in feces at room temperature may vary from one day to several weeks.
- It is readily inactivated by heat (55°C for 30 minutes). Molar $MgCl_2$ protects the virus against heat inactivation. Milk or ice cream also provides such protection.
- Formaldehyde and oxidising disinfectants destroy the virus. Chlorine at 0.1 ppm can destroy purified polio. However, higher concentrations are required to destroy the virus in sewage water, organic matter and in feces. Phenolic disinfectants are not effective.
- Poliovirus does not survive lyophilisation.

Pathogenesis

Transmission

Natural infection occurs only in humans. Experimentally, chimpanzees and cynomolgus monkeys may be infected by the intracerebral, intraspinal or oral routes.

The virus enters by the oral route through ingestion of food and water contaminated with human feces. The virus colonises the nasopharynx and multiplies initially. It may be found in the throat or feces in the initial phases. Then, it passes down to Peyer's patches and the epithelial cells of the alimentary canal lymphatic tissues.

Spread

After gaining entry, the virus then spreads to the regional lymph nodes and enters the bloodstream (**primary viremia**). After further multiplication in the reticuloendothelial system, the virus enters the bloodstream again (**secondary viremia*)*** and is carried to the spinal cord and brain. The virus

can pass along the axons of the peripheral nerves to the central nervous system (CNS). In poliomyelitis following tonsillectomy, neural transmission to CNS can occur. In the **central nervous system**, the virus multiplies selectively in the neurons and destroys them. The earliest change is the degeneration of **Nissl bodies** (**chromatolysis**). Nuclear changes follow. When degeneration becomes irreversible, the necrotic cell lyses or is phagocytosed by leucocytes or macrophages. Degenerative lesions are mostly in the **anterior horns of the spinal cord** (Fig. 46.2), causing **flaccid paralysis**, but the posterior horns and intermediate columns may also be involved to some extent. In some cases, encephalitis may occur, involving the brainstem and extending up to the motor and premotor areas of the cerebral cortex.

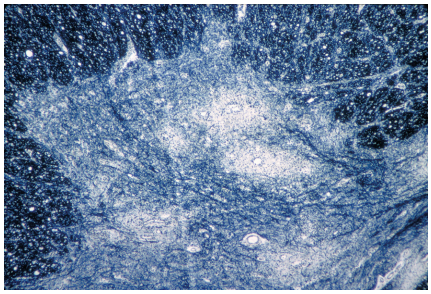


Fig. 46.2 Photomicrograph of a spinal cord tissue sample in the region of the anterior horn revealing polio type III degenerative changes (*Source:* Public Health Image Library, ID 2757 / Dr. Karp, Emory University / CDC)

Clinical Features

Infection is, mostly, asymptomatic. The ratio of subclinical to clinical infections has been stated to be 100 or 1,000 to 1. The outcome of infection is influenced by the virulence of the infecting strain, the infective dose and the age of the individual (adults being more susceptible than children). The following factors may influence the incidence of paralysis:

- Pregnancy carries an increased risk of paralysis, perhaps due to the associated hormonal changes.
- Poliovirus type 1 is responsible for most epidemics of paralytic poliomyelitis. Type 3 also causes epidemics to a lesser extent. Type 2 usually causes inapparent infections in western countries but, in India, paralysis due to type 2 is common. Immunity is type-specific but there is a significant amount of cross-protection

between types 1 and 2, and types 2 and 3 and little or none between types 1 and 3.

Infection may manifest in several forms:

- **Inapparent infection** Following exposure to the poliovirus, 90–95 per cent of susceptible individuals develop only inapparent infection, which causes seroconversion alone. It is only in 5–10 per cent that any sort of clinical illness results. The incubation period is about 10 days on an average, but may range from four days to four weeks.
- **Abortive poliomyelitis or minor illness** The earliest manifestations are associated with the phase of primary viremia consisting of fever, headache, sore throat and malaise lasting 1–5 days.
- **Paralytic poliomyelitis or major illness** If the infection progresses, the minor illness is followed 3–4 days later by the major illness. The fever returns (**biphasic fever**), along with headache, stiff neck and other features of meningitis. This marks the stage of viral invasion of the central nervous system (*Clinical case*). Paralytic polio is more common in older individuals, pregnant women and persons who perform strenuous physical activity. Tonsillectomy predisposes a person to bulbar poliomyelitis; IM injection during the course of febrile polio phase increases the risk of paralysis.
- In those proceeding to paralytic poliomyelitis**, flaccid paralysis** develops. Paralysis is focal in distribution initially, but spreads over the next 3–4 days. Depending on the distribution of paralysis, cases are classified as **spinal, bulbar** or **bulbospinal.** The mortality range is 5–10 per cent and is mainly due to respiratory failure. Recovery of the paralysed muscles takes place in the next 4–8 weeks and is usually complete after six months, leaving behind varying degrees of residual paralysis (Fig. 46.3). Rarely, myocarditis or lymphatic hyperplasia may occur.

Fig. 46.3 Polio-afflicted individual with paralysis of the right lower limb (*Source:* Public Health Image Library, ID 134 / CDC /NIP / Barbara Rice / CDC)

- **Non-paralytic poliomyelitis** Sometimes, the disease does not progress beyond the stage of aseptic meningitis.

Post-polio syndrome occurs years later (20–40 years) due to the loss of function of motor neurons which had compensated for the loss in the initial infection. This is seen more often in women, after the initial infection. It presents as pain, fasciculation, atrophy, fatigue and weakness.

Laboratory Diagnosis

Laboratory confirmation of polio is done by the following methods:

- Culture
- Intratypic differentiation
- Genome sequencing
- Serology

1. **Specimen** Throat swab and feces (rectal swabs), along with blood and CSF are collected for laboratory diagnosis.

Examination of CSF reveals lymphocytic pleocytosis, normal glucose and normal-to-slightly elevated protein. About 1% patients present with aseptic meningitis.

2. **Virus isolation** Isolation of the virus in tissue culture is the best method for specific diagnosis and typing the strain.

The virus grows readily in tissue cultures of primate origin. Primary monkey kidney cultures or continuous cultures from human tissues are used

for the isolation of the virus from clinical material and vaccine production. The infected cells round up and become refractile and pyknotic. Eosinophilic intranuclear inclusion bodies may be demonstrated in stained preparations. **Well-formed plaques develop in infected monolayers.**

- The virus can be isolated from **blood** during primary viremia, 3–5 days after infection, before neutralising antibodies appear. However, this is of little practical importance.
- The virus can be isolated from the **throat** within 14 days of the onset of the disease.
- Virus isolation from **feces** is usually possible in over 80 per cent of patients in the first week, 50 per cent till the third week and 25 per cent till the sixth week. As fecal excretion may be intermittent, the best results are obtained by testing fecal samples collected on two separate days, as early in the illness as possible. Prolonged fecal excretion may be seen in the immunodeficient. Carrier stages do not occur.
- Unlike other enteroviruses, the poliovirus can seldom be isolated from the cerebrospinal fluid (CSF) but can be obtained from the **spinal cord and brain**, postmortem.

Virus growth is indicated by typical cytopathic effects in 2–3 days. The viruses are identified by neutralisation tests with pooled and specific antisera. It must be remembered that the mere isolation of the poliovirus from feces does not constitute a diagnosis of poliomyelitis as symptomless infections are common. **Virus isolation must be interpreted along with clinical and serological evidence**.

Real-time reverse transcription PCR is used for intratypic differentiation using viruses isolated in culture as the starting material.

3. Molecular diagnosis

- **Reverse transcriptase PCR** PCR-based tests have improved the demonstration of viral RNA in CSF.
- **Sequencing** In a population, three types of strains may be in circulation: wild virus, oral polio vaccine (OPV) virus strain and

vaccine-derived polioviruses **(VDPVs)**. These can be differentiated by sequencing. Partial genome sequencing is used to confirm the poliovirus genotype and determine its likely geographic origin.

4. **Serodiagnosis** Antibody rise can be demonstrated in **paired sera** by **neutralisation** or **complement fixation** tests. Antibodies appear soon after the onset of paralysis. Neutralising antibodies appear early and persist for life. In the CF test, antibodies to the C antigen appear first and disappear early, while anti-D antibodies take some weeks to appear after infection but last for about five years.

Immunity

Immunity in poliomyelitis is **type-specific and permanent**. Humoral immunity provided by circulating and secretory neutralising antibodies is responsible for protection against poliomyelitis. The IgM antibody appears within a week of infection and lasts for about six months. The IgG antibody persists for life. Antibodies cannot protect once the infection spreads to the CNS. **Hence vaccination must be prior to the onset of CNS symptoms**. Secretory IgA in the gastrointestinal tract provides mucosal immunity, preventing intestinal infection and virus shedding. Breast milk containing IgA antibody protects infants from infection. The virus also induces cell-mediated immunity (CMI) but its effect is doubtful.

Immunoprophylaxis

Passive immunisation by the administration of human gammaglobulin is of little value.

Active immunisation

Salk's killed vaccine [injectable polio vaccine (IPV)]

This comprises formalin-inactivated types 1–3 poliovirus, grown in monkey kidney tissue culture. Inactivation with formalin (1:4,000) is done at 37°C for 12–15 days. Stringent tests are carried out to ensure complete inactivation and freedom from extraneous agents.
Tests for safety and potency are done before issuing them for use.

A setback to Salk candidate vaccine occurred in 1955, when over 100 cases of paralytic poliomyelitis occurred in the vaccinated persons and their contacts. The '**Cutter incident**' (so called after the manufacturer of the particular vaccine) occurred due to incomplete inactivation of viruses. This led to the introduction of further safeguards. The vaccine, after these modifications, has been completely safe.

Vaccination schedule The killed vaccine is given by injection, hence called **inactivated** or **injectable polio vaccine** (IPV). Three doses are given 4–6 weeks apart, which is followed by a booster six months later.

- The **first dose** should be given to babies above six months of age to ensure that maternal antibodies do not interfere.
- **The second dose**—an enhanced potency IPV produced in human diploid cells induces better seroconversion following two subcutaneous doses 4–8 weeks apart.
- **A third dose** may be given 6–12 months later.
- **Booster doses** help to sustain immunity; they can be administered every 3–5 years after the third dose.

Sabin's live attenuated vaccine [oral polio vaccine (OPV)]

Three live polio vaccines were developed independently by Koprowski, Cox and Sabin. All three were used initially, but **currently, Sabin's attenuated strains are employed**. The attenuated virus immunises by active multiplication. It stimulates systemic production of IgM, IgG and secretory IgA in the intestine, producing local immunity.

The use of monoclonal antibodies specific for vaccine strains, oligonucleotide fingerprinting and nucleic acid sequencing help to differentiate wild type from vaccine type strains.

Vaccination schedule Live polio vaccine is administered orally and is, therefore, known as the **oral polio vaccine (OPV).** Very stringent precautions are taken to ensure freedom from extraneous agents like SV40 and the B virus in cell cultures used to prepare vaccines. **Monovalent or trivalent forms** in pleasantly flavoured syrups are prepared. $MgCl_2$ or sucrose stabilises the vaccine against heat inactivation, particularly in tropical conditions.

Bivalent OPV (**bOPV containing types 1 and 3 Sabin viruses**) has been licensed and used in some settings since December 2009. Trivalent OPV has been replaced with bivalent OPV from April 2016 as

per the World Health Organization module. OPV containing serotype 2 monovalent is kept for emergency use.

Dosage Four doses are given at 4–8-week intervals.

There has been much controversy about the relative merits of killed and live vaccines.

Safety Both vaccines are safe. It has been suggested that the attenuated strains tend to acquire neurovirulence on serial enteric passage, as may happen following vaccination. A few cases of vaccine-induced poliomyelitis have been reported but the incidence is so low that the risk is negligible. However, it assumes importance where the disease has been eradicated by immunisation. OPV is not safe in immunodeficient or immunosuppressed subjects but the killed vaccine is harmless.

Vaccine-derived poliovirus (VDPV)

On very rare occasions, OPV can lead to **vaccine-associated paralytic polio (VAPP)**. It may also be caused by a strain of poliovirus that has genetically changed in the intestine from the original attenuated vaccine strain contained in OPV. This strain is known as vaccine-derived poliovirus (VDPV). The longer it is allowed to survive, the more genetic changes it undergoes. In rare instances, the vaccine-virus can genetically change into a form that can paralyse the individual who gets infected—the strain is known as a circulating vaccine-derived poliovirus [**circulating VDPV (cVDPV)**].

Two other VDPVs are:

- **Immune-deficiency associated VDPV (iVDPV)** VDPVs isolated from persons with evidence of primary immunodeficiency.
- **Ambiguous VDPV (aVDPV)** A VDPV isolate from individuals or from environmental samples, without evidence of circulation and from individuals with no known immunodeficiency.

Efficiency

A full course of killed vaccine induces a satisfactory immune response in a healthy child (Fig. 46.4).

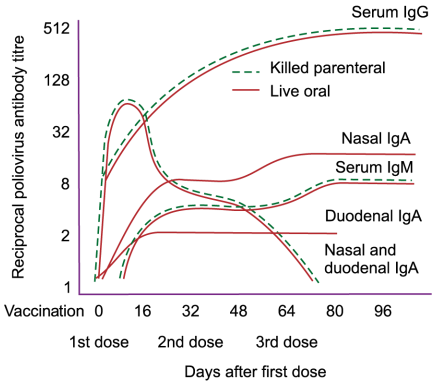


Fig. 46.4 Serum and secretory antibody response to 3 doses of OPV or 3 doses of IPV in a healthy child

Issues related to OPV failure in the tropics

- Interference by other enteroviruses is common in the tropics (experimentally, it has been shown that coxsackie B viruses may interfere with the poliovirus, while coxsackie A may be synergistic).
- Frequent diarrheal diseases prevent colonisation by the vaccine virus.
- Breastfeeding immediately before or after the vaccine may lead to the failure of the vaccine—the virus may be neutralised by antibodies in breast milk or other inhibitory agents in the intestinal secretions.
- An inhibitor of poliovirus has been identified in saliva. This can be countered by horse antiserum to human gamma globulin. Because of the poor rate of seroconversion after oral immunisation in India and other tropical areas, primary immunisation with killed vaccines (given along with the triple antigen) has been proposed. This may be followed by live oral vaccines for achieving intestinal immunity.

Key advantages of OPV

1. ***Ease of administration*** OPV is obviously preferable to killed vaccines given by injection. However, an advantage of the killed vaccine is that it can be administered along with the DPT vaccine as a quadruple vaccine.

2. ***Economy*** The live vaccine is much more economical. This is an important aspect of mass vaccination campaigns in the developing countries.
3. ***Nature of immunity*** This is perhaps the most important difference between the two. Killed vaccine induces only systemic antibody response. There is no **intestinal immunity**; even in the vaccinated, infection with a wild strain may lead to intestinal multiplication and dissemination of the virus. The individual alone is protected by the circulating antibodies. Live vaccines, on the other hand, also induce local immunity in the gut so that wild viruses are unable to multiply in the intestines and be shed. Hence it protects the individual and the community, producing herd immunity.
4. ***Duration of immunity*** Immunity following a killed vaccine may need to be maintained by booster doses periodically, while immunity following a live vaccine resembles natural active immunity in lasting longer.
5. ***Use in epidemics*** Community-wide administration of OPV, ideally a monovalent vaccine of the same type as that causing the epidemic, early during an epidemic of paralytic poliomyelitis can stop the epidemic. This has been successfully practised in different parts of the world.
6. ***Spread of vaccine virus in the community*** The tendency of the vaccine virus to spread naturally in the community, especially among children, is a disadvantage in developed countries.

The salient differences between oral and injectable polio vaccines are depicted in Table 46.1.

Table 46.1 Salient differences between oral and injectable polio vaccine

Characteristics of polio vaccines	Oral polio vaccine	Injectable polio vaccine
Nature	OPV is composed of live, attenuated polioviruses that are grown in non-human cells—Sabin 1, 2 and 3 Bivalent OPV drops consisting of attenuated	Inactivated (killed) poliovirus strains of all three poliovirus—types 1, 2 and 3 Combined with other

	strains of types 1 and 3	routine vaccine antigens like diphtheria, tetanus, whole-cell or acellular pertussis, hepatitis B, or *Haemophilus influenzae* type b
Virulence and transmission	Have negligent neurovirulence and transmissibility	Non-transmissible and are avirulent as they are killed
Route of administration	Oral	IM or subcutaneous
Immunity	Mucosal immunity along with humoral immunity Produces herd immunity in the community through excretion by vaccinated children	Systemic humoral immunity Does not produce herd immunity
Drawbacks		
Administration	Easy to administer; additional doses of OPV on all pulse polio days for children till 5 years	Cannot be given as a mass schedule
Genetic change	Can genetically revert to iVDPV (immunodeficiency-related vaccine-derived poliovirus), aVDPV (ambiguous vaccine-derived poliovirus) or cVDPV(circulating vaccine-derived poliovirus)	Cannot revert
Safety	Rarely, may give rise to VAPP	There is no such danger

Vaccine virus may even be beneficial and may help extend the vaccine coverage in countries where the wild virus is endemic. Ideally, however, it is desirable to vaccinate the whole community at one time so that natural dissemination is prevented. The strategy of administering the vaccine to all

children in a region on the same day (**pulse immunisation**) has been found to be useful in developing countries.

Eradication of poliomyelitis

It is believed that the eradication of poliomyelitis is possible if global immunisation is effectively employed. A successful polio immunisation programme relies on effective planning with measurable outcomes including vaccine procurement and supply, cold chain and logistics, health worker training, communications and social mobilisation. The end game strategic plan of the **Global Polio Eradication Initiative (GPEI)** is to deliver a sustained, polio-free world (planned to be implemented between 2013–2018). The strategy is as follows:

i) Detection and interruption of poliovirus transmission: wild virus interruption leading to outbreak interruption
ii) Immunisation strengthening and withdrawal of OPV: introduce IPV, switch to bOPV and withdraw OPV
iii) Containment and certification: contain polio and certify interruption of transmission
iv) Legacy planning: consultation and mainstream polio functions

The end game strategic plan of the Global Polio Eradication Initiative (GPEI) has been updated as Polio Endgame Strategy 2019–2023, eradication, integration, certification and containment (http://polioeradication.org/wp-content/uploads/2019/06/english-polio-endgame-strategy.pdf).

Pulse polio immunisation programme (PPI)

The Government of India has used this programme to achieve the goal of eradicating poliomyelitis. It includes mass administration of OPV on a single day to all children aged 0–5 years in the community, irrespective of whether they have been vaccinated or not, through **national universal immunisation programme (UIP).** It does not replace the UIP. The strategy was to give two rounds of vaccination at an interval of 4–6 weeks during the low polio transmission period in winter (November to February). The World Health Organization declared **India free of polio** on 27 March 2014.

Epidemiology

Poliomyelitis is an exclusively human disease. The only source of the virus are humans—the patient or, much more commonly, the symptomless carrier. Patients shed the virus in feces for varying periods, about 50 per cent for three weeks, and a small proportion for 3–4 months. No permanent carriage occurs. However, the virus may persist in the environment (sewage) for up to six months. Virus shed in throat secretions during the early part of the disease may also be a source of infection.

Global and India's status on polio

The WHO has declared major continents free of polio except a few African and Asian countries. India has also been declared polio-free as there has not been any report of wild poliovirus infection for the last five years. This has been possible due to active polio eradication programme, sustained vaccination through pulse polio and mop-up programmes. India has actively participated in the GPEI for a polio-free world.

There are a few countries which still report cases, notably Pakistan and Afghanistan with wild poliovirus infections being reported while the Democratic Republic of Congo, Nigeria and Papua New Guinea report cVDPV infection as of 2019 (February). (http://polioeradication.org/polio-today/polio-now/this-week/)

COXSACKIEVIRUSES

The characteristic feature of this group is its ability to infect suckling but not adult mice. Also, only few types can grow in cell cultures. Based on the pathological changes produced in suckling mice, coxsackieviruses are classified into two groups: A and B.

Properties

Coxsackieviruses are typical enteroviruses. Two groups, A and B, are known. By neutralisation tests, group A viruses are classified into 24 types and group B into 6 types. Their properties are as follows:

Group A viruses (24 serotypes)

Following inoculation in suckling mice, group A viruses produce generalised myositis and flaccid paralysis, leading to death within a week.

Coxsackie A23 is the same as echovirus 9, and coxsackie A24 the same as echovirus 34. Some coxsackieviruses (A7, 20, 21, 24 and B1, 3, 5, 6) agglutinate human or monkey erythrocytes.

Group B viruses (6 serotypes)

Following inoculation in suckling mice, they produce patchy focal myositis,

spastic paralysis, necrosis of brown fat and, often, pancreatitis, hepatitis, myocarditis and encephalitis.

All types in group B share a common complement-fixing antigen.

Clinical Features

Coxsackieviruses produce a variety of clinical syndromes in humans, ranging from trivial to fatal infections.

The types that have been recognised are presented in Table 46.2.

Table 46.2 Clinical syndromes commonly caused by enteroviruses

Syndrome	Poliovirus	Cox-sackie A	Cox-sackie B	ECHO	New entero-virus types
Paralysis	1, 2, 3	7	–	–	–
Aseptic meningitis	1, 2, 3	7, 9, 23	1–6	Several (2, 3, 4, 6, 7, 9, 11, 14, 16, 17, 18, 19, 25, 33)	71
Encephalitis	1, 2, 3	9	3–6	6, 9, 17, 19	71
Fever with rash	–	9, 16, 23	–	4, 6, 9, 16	–
Hand, foot and mouth disease	–	5, 10, 16	–	–	71
Herpangina	–	1–6, 8, 10	–	–	–
Upper respiratory infection	–	21	–	11, 20	–
Pneumonitis, bronchiolitis	–	–	–	–	68
Bornholm disease	–	–	1.5	–	–
Myocarditis, pericarditis	–	–	1.5	–	–
Acute hemorrhagic conjunctivitis	–	24	–	–	70

Group A

- **Herpangina** (vesicular pharyngitis) is a common clinical manifestation of coxsackie group A infection in children. It is a severe febrile pharyngitis, with headache, vomiting and pain in the abdomen. The characteristic lesions are small vesicles, on the

fauces and posterior pharyngeal wall, which break down to form ulcers.

- **Aseptic meningitis** may be caused by most group A and all group B viruses. A maculopapular rash may be present. The disease may sometimes occur as epidemics. Type A7 had caused outbreaks of paralytic disease in Russia, Scotland and elsewhere, the virus for a time having been erroneously referred to as poliovirus type 4.
- **Hand, foot and mouth disease (HFMD**) is an exanthematous fever mainly affecting young children; it is characterised by clusters of papulovesicular lesions on the skin and oral mucosa. It occurs as sporadic cases and as outbreaks. Coxsackie A16, A9 and B1–3 were common causative agents initially. Subsequently, Enterovirus 71 caused extensive epidemics with serious complications like aseptic meningitis, encephalitis, flaccid paralysis, pulmonary hemorrhage, with many fatalities, particularly in East Asia, from Taiwan to Singapore. HFMD in now an important emerging disease.
- **Minor respiratory infections** resembling the common cold may be caused by A10, A21, A24 and B3.

Group B

- Epidemic myalgia or **pleurodynia,** also known as **Bornholm disease** (first described on the Danish island of Bornholm), is a febrile disease with a sharp piercing pain in the chest and abdomen, caused by group B viruses. The disease may occur sporadically or as epidemics.
- **Myocarditis** and **pericarditis** in the newborn, associated with high fatality, may be caused by group B viruses. The disease may sometimes occur in older children and adults as well.
- **Juvenile diabetes** has been claimed to be associated with coxsackie B4 infection but a causal role for this virus has not been established.
- **Orchitis** due to coxsackievirus has also been reported.

- **Transplacental and neonatal transmission** have been demonstrated with coxsackie B viruses; they result in a serious disseminated disease that may include hepatitis, meningoencephalitis and adrenocortical involvement.
- Type B viruses have been associated with a condition called **post-viral fatigue syndrome** but neither the condition nor the association has been clearly defined.

Laboratory Diagnosis

1. **Animal inoculation** Coxsackievirus can be isolated by inoculation in suckling mice. Inoculation is usually made by the intracerebral, subcutaneous and intraperitoneal routes. Adult mice are not susceptible. Suckling hamsters can also be infected experimentally.

All coxsackie B viruses grow well in monkey kidney tissue cultures, while only types 7 and 9 of group A grow well in this. The A21 virus grows in HeLa cells.

2. **Virus isolation** The virus may be isolated from lesions or feces by inoculation into suckling mice. Identification is by studying the histopathology in infected mice and by neutralisation tests.

3. **Tissue culture** This is not useful as all serotypes do not grow well in cell lines.

Due to the existence of several antigenic types, **serodiagnosis** has not been of much use.

Epidemiology

Like other enteroviruses, the coxsackievirus primarily inhabits the alimentary canal and spreads by the fecal–oral route. Coxsackie B virus epidemics tend to occur every 2–5 years. Infants are most commonly affected. **Vaccination is not practicable as there are several serotypes and immunity is type-specific.** Many enteroviruses share similarity with coxsackieviruses. They may occur together in the environment, sewage or gut of the same human host.

ECHOVIRUSES

ECHO virus could not be associated with any particular clinical disease, hence they were called orphans. They are known as **enteric cytopathogenic human orphan (ECHO) viruses**, or more commonly, 'echoviruses'.

Properties

Echoviruses resemble other enteroviruses in their properties. They have been classified into 34 serotypes by neutralising antibodies.

Some echoviruses (types 3, 6, 7, 11, 12, 13, 19, 20, 21, 24, 29, 30 and 33) agglutinate human erythrocytes. Hemagglutination is followed by elution, rendering the cells inagglutinable by echo or coxsackieviruses but not by myxoviruses.

Types 10 and 28 have been removed from the group, the former becoming a reovirus and the latter a rhinovirus.

Clinical Features

Most infections by echovirus are asymptomatic or may resemble those produced by coxsackieviruses. Fever with rash and aseptic meningitis occurs sometimes as epidemics caused by types 4, 6, 9, 16, 20, 28 and 30. **Echoviruses constitute perhaps the most common cause of aseptic meningitis.** They have frequently been isolated from **respiratory disease** in children (types 1, 11, 19, 20 and 22) and **gastroenteritis (type 18)** but their causative role has not been proved. Occasional cases of paralysis and hepatic necrosis have also been reported.

Laboratory Diagnosis

All echoviruses grow well in human and simian kidney cultures, producing cytopathic effects. Feces, throat swabs or CSF may be inoculated into monkey kidney tissue cultures and virus growth detected by cytopathic changes. Serological diagnosis is not practicable except in cases of epidemics where the causative serotype has been identified.

Epidemiology

Like other enteroviruses, echoviruses primarily inhabit the alimentary tract and are spread by fecal transmission through contaminated drinking water. Epidemics may occur, especially in summer. Vaccination has not been

attempted.

NEW ENTEROVIRUS TYPES

Enterovirus 68–71

New enteroviruses include types 68–71. Type 68 was isolated from pharyngeal secretions of children with pneumonia and bronchitis. Type 69 is not associated with any human disease. Type 70 causes acute hemorrhagic conjunctivitis. EV-71, originally isolated from cases of meningitis and encephalitis, causes many other syndromes, including HFMD. Additional new enteroviruses include:

- EV-79–88
- EV-97
- EV-100–116

Acute Hemorrhagic Conjunctivitis

A pandemic of acute hemorrhagic conjunctivitis occurred in 1969 and affected most countries. The incubation period of the causative organism was found to be about 24 hours with swelling, congestion, watering and pain in the eyes. Subconjunctival hemorrhage is a characteristic feature of this type of conjunctivitis. There is transient corneal involvement but recovery is usually complete in 3–7 days. **Radiculomyelopathy has been reported from India as a complication**. Sometimes, it leads to paralysis resembling poliomyelitis.

It is caused by enterovirus type 70 (**EV-70**). This virus grows only on human embryonic kidney or HeLa cell lines for primary isolation but can be adapted to grow on monkey kidney cells. Coxsackievirus type A24 also produces the same disease. Both these viruses show intratypic antigenic differences. Enterovirus 71 has been associated with fatal meningitis, encephalitis and paralysis.

RHINOVIRUSES

Common cold is probably the most common infectious disease of humans. The **common cold virus** was isolated by Tyrrell and his colleagues (1960).

Currently, more than 100 serotypes are known, classified through neutralisation tests.

Properties

Rhinoviruses resemble other picornaviruses in size and structure. They are inactivated below pH 6, inactivation being complete at pH 3. They are relatively stable at 20–37°C and may remain viable on fomites for days. Some serotypes survive for one hour at 50°C.

Pathogenesis

The virus attaches to receptors on nasal ciliated epithelial cells, enters and replicates within them, spreading to other cells. The cilia and cells are damaged and the epithelium is subjected to secondary bacterial infection. Local inflammation and cytokines may be responsible for the symptoms of common cold. Interferon production occurs early and specific antibody appears in nasal secretions. Immunity to rhinovirus is type-specific. Rhinoviruses can causes serious complications like pneumonia and respiratory distress syndrome in the elderly with underlying co-morbid conditions.

Laboratory Diagnosis

Rhinoviruses can be grown in tissue cultures of human or simian origin with cytopathic changes if good oxygenation (achieved by rolling), low pH (around 7) and low temperature (33°C) are provided. Isolation of the virus may be done from nasal or throat swabs collected early in the infection, in human cell cultures, preferably MRC5 or W138 strains. Growth as evidenced by cytopathic effect (CPE) may take two weeks to appear. Due to the large number of serotypes, serology is not feasible for diagnosis.

Epidemiology

The common cold is an infectious disease seen across the globe. It is transmitted by droplets. Hand-to-hand contact followed by self-inoculation of conjunctival or nasal mucosa appears to be an important mode of transmission. The multiplicity of serotypes makes vaccination impossible. Moreover, the common cold is a syndrome produced not only by

rhinoviruses but also by a variety of other groups such as respiratory syncytial, corona, coxsackie, echo, adeno, influenza and parainfluenza viruses.

Key points

- Picornaviridae is a family of non-enveloped viruses, 25–30 nm in diameter (among the smallest of viruses), with icosahedral symmetry and a single-stranded RNA genome. The enteroviruses and rhinoviruses are important human pathogens.
- *Enterovirus* is a genus of the family Picornaviridae, comprising 3 polioviruses, 21 coxsackie A and six coxsackie B viruses, 29 echoviruses and enteroviruses types 68–71.
- Poliomyelitis is caused by three types of polioviruses, with type I producing the most severe disease. Only humans are naturally infected.
- Polioviruses can be isolated in tissue culture. Viral RNA can be detected by RT-PCR in throat swabs or samples of feces.
- Poliomyelitis is prevented by an inactivated (killed) injectable vaccine (Salk vaccine) or an attenuated live, orally-administered vaccine (Sabin vaccine), which confers immunity by raising neutralising antibodies.
- Pulse Polio Immunisation Programme (PPI) helps increase herd immunity.
- Coxsackie A viruses are associated with hand, foot and mouth disease and meningitis; Coxsackie B viruses are associated with Bornholm disease, meningitis, pericarditis and myocarditis. For both, only a few serotypes grow in cell culture, and suckling mice are the most susceptible laboratory animals.
- Echoviruses are commonly found in the feces of healthy children. They are now known to cause meningitis, exanthema and severe neonatal diseases. They grow readily in cell culture, but rarely cause any lesions in suckling mice.
- Rhinoviruses have worldwide distribution and cause the common cold. There are over 100 antigenic types and cross-protection among them is limited, allowing repeated infections to occur.
- There are no specific antiviral agents against *Enterovirus* unlike some other viral agents causing human infection.

Questions

Essay

1. Describe the etiology, modes of transmission, pathogenesis and laboratory diagnosis of polio.

Short Answers

1. Prophylaxis against polio
2. Pulse polio immunisation programme

3. Enteroviruses of public health importance and the diseases caused by them
4. Oral polio vaccine and criteria for its attenuation
5. Inactivated polio vaccine
6. Coxsackievirus
7. Acute hemorrhagic viral conjunctivitis
8. Rhinoviruses
9. Types of poliovirus and their epidemiology
10. Eradication of polio

Part IV: Virology

Chapter 47

Reoviridae

Chapter navigation

- *Reovirus*
- *Orbivirus*
- *Rotavirus*
- *Calicivirus* and other diarrheagenic viruses

A group of RNA viruses of the family **Reoviridae** derives its name from the prototype virus which was known as the respiratory enteric orphan (REO) virus because it was frequently isolated from the respiratory and enteric tracts but was not associated with any disease. Members of this family are double-shelled icosahedral viruses, 55–57 nm in diameter. The genome consists of **double-stranded RNA** in 10–12 pieces, a feature unique among animal viruses. They are non-enveloped and resistant to lipid solvents. The family contains three genera: *Reovirus, Orbivirus* and *Rotavirus*. While the first two are not associated with human infections, *Rotavirus* is a major viral agent responsible for diarrhea in children and adults. It accounts for several childhood deaths in developing countries.

REOVIRUS

The genus *Reovirus* contains three mammalian serotypes (1, 2 and 3). Reoviruses have not been proved to be the causative agents of any human disease.

ORBIVIRUS

This group of viruses consists primarily of animal pathogens. These viruses are responsible for disease in horses and cattle. They cause bluetongue disease in sheep and hemorrhagic fever in deer.

ROTAVIRUS

Rotavirus is the major cause of diarrhea in infants. It resembles cartwheels with short spokes radiating from a wide hub to a clearly defined outer rim. The name is derived from the Latin word *rota*, meaning 'wheel'. The complete or **double-shelled virus** measures about 70 nm in diameter and has a smooth surface. It has a double-stranded RNA with the genome in 11 segments. The incomplete or **single-shelled virus** is smaller, about 60 nm, with a rough surface that has lost the outer shell (Fig. 47.1). 'Empty' particles without the RNA core are also seen.

Identified by Bishop and colleagues (1973) in Melbourne, rotaviruses are now recognised as the **most common cause of diarrheal disease in infants and children**.

Outbreaks of rotavirus diarrhea in older children and adults have been reported from different parts of China. This rotavirus is called **adult diarrhea rotavirus (ADRV)**.

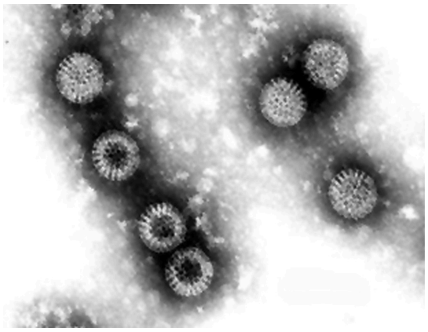


Fig. 47.1 Electron micrographic appearance of rotavirus—double-shelled virus and single-shelled virus showing cartwheel appearance

Classification

Rotaviruses have been classified into antigenic groups (A to G) based on the antigenic epitopes of internal structural protein (VP6). Group A strains, which cause the majority of human infections in children, have been classified into **subgroups (I and II)** which can be detected by ELISA and immune electron microscopy, and further **serotypes (1, 2, 3, etc.)** which can be detected by neutralisation tests. Outer viral capsid proteins VP4 and VP7 carry epitopes important for neutralisation. The predominant antigen is the glycoproteinVP7.

ADRV strains belong to Group B. By polyacrylamide gel electrophoresis, rotaviral strains can be classified into several electrophoretypes based on the patterns of migration of the viral RNA.

Human rotavirus does not grow readily in cell cultures but some strains have been adapted for serial growth in tissue cultures. Rotavirus growth is facilitated by trypsin treatment and rolling of tissue cultures. As calf and simian viruses grow readily in cell cultures, they have been used as antigens for serological studies.

Rotaviruses are a class of viruses causing diarrhea in the young of many animals and some birds. The human rotavirus is related to the viruses of epidemic diarrhea of infant mice (EDIM), Nebraska calf diarrhea and the simian virus SA11. All rotaviruses share common antigens. Though the viruses are generally species-specific, interspecies infection can be induced experimentally. Human rotavirus infection has been transferred to piglets, calves and monkeys. It is not known whether human infection can be caused by animal rotaviruses.

Pathogenesis

Infection is by the fecal–oral route. The incubation period is 2–3 days. Vomiting and diarrhea occur with little or no fever. Stools are usually greenish-yellow or pale, with no blood or mucus. The disease is self-limiting and recovery occurs within 5–10 days. Mortality is low.

Laboratory Diagnosis

Most human rotaviruses of Group A can be cultured if they are pre-treated with the proteolytic enzyme, trypsin, in cell cultures with low levels of trypsin.

Serological techniques IgM and IgG antibodies can be demonstrated in the blood of infected children by ELISA. Rotaviruses share a common group antigen situated in the inner capsid layer. The virus can be detected in stool filtrates by enzyme immunoassays (EIA) and immune electron microscopy (IEM). Genotyping can be done by polymerase chain reaction (PCR), which is a sensitive detection method.

Epidemiology

Rotaviruses are the commonest cause of diarrhea in infants and children the world over and account for about half the cases of children hospitalised for diarrhea. Infections occur throughout the year but are predominant in the winter months, when the virus may be detected in most patients. It sometimes produces large epidemics of diarrhea in winter. Rotavirus diarrhea is usually seen in children below the age of five years but is most frequent between 6 and 24 months of age. Infection is not infrequent in neonates but they seldom develop diarrhea because of maternal passive immunity. By the age of five years, most children have had clinical or subclinical infection, so rotavirus diarrhea is very uncommon in older children and adults.

Treatment

Rehydration is all the treatment needed. There are no antiviral agents.

Prophylaxis

Rotavirus vaccines are in use in 23 countries. Commonly, the **RotaTeq** (RV°) vaccine is used. India has also recently introduced an indigenously developed rotavirus vaccine, **Rotarix (RV1**). Both vaccines are given orally.

OTHER DIARRHEAGENIC VIRUSES

Besides rotaviruses, the following viruses are known or suspected to cause diarrheal disease.

Calicivirus

Calicivirus is a group of important viruses that cause gastroenteritis in humans. The most important virus in this group is the Norovirus whose prototype is the Norwalk virus.

Norwalk virus A 27 nm virus was shown to be responsible for an epidemic of gastroenteritis affecting school children and teachers in Norwalk, Ohio, in 1972. Serological surveys have shown that infection with Norwalk virus is widespread in many countries. Epidemics of Norwalk virus diarrhea associated with the consumption of raw oysters have been reported from Australia and America.

The virus can be demonstrated in feces by electron microscopy. Antibodies to the virus can be detected by immune electron microscopy and radioimmunoassay. It has been included in the family Caliciviridae, which consists of small, round RNA viruses, 22–30 nm in
size, many of which have been reported from diarrheal feces. The name is derived from the presence of 32 cup-shaped depressions on the virus surface (from *calyx*, meaning cup).

Adenovirus

Several outbreaks of diarrhea in children have been associated with the presence of large numbers of adenoviruses in feces. These can only be grown with difficulty in tissue culture. They have been designated types 40 and 41. Adenovirus-associated diarrhea is seen more often in the summer months.

Astrovirus

These star-shaped, 28 nm isometric particles have been associated with some epidemics of diarrhea in children. Similar viruses have also been identified in lamb and calf diarrhea.

Coronavirus

These viruses are well-established causes of acute diarrhea in calves, piglets and dogs. They have been observed in human feces also and diarrhea is often observed in coronavirus respiratory infections, but their relation to diarrhea is uncertain. However, diarrhea is often observed in coronavirus respiratory infections.

Key points

- Reoviridae is a family of non-enveloped RNA viruses with a double layer of capsomeres arranged in concentric spheres around the nucleoprotein. There are three genera: *Reovirus*, *Orbivirus* and *Rotavirus*.
- *Rotavirus* is a common cause of human and animal gastroenteritis.
- Viruses that are important causes of diarrhea are rotaviruses, the Norwalk virus, certain adenoviruses and coronaviruses, and astroviruses.

Questions

Essay

1. List the viruses that cause diarrhea. Describe rotavirus diarrhea in children. Mention the prophylaxis of rotavirus diarrhea.

Part IV: Virology

Chapter 48

Orthomyxovirus

Chapter navigation

- Influenza
  - Nomenclature systems
  - Classification
  - Antigenic variations
  - Epidemics and pandemics
  - Vaccines
- Avian influenza
- Swine influenza

INTRODUCTION

The name Myxovirus was used originally for a group of enveloped RNA viruses characterised by their ability to adsorb onto mucoprotein receptors on erythrocytes, causing hemagglutination. The name referred to the affinity of the viruses to mucins (from *myxa*, meaning mucus). They are now classified into two separate families: **Orthomyxoviridae**, consisting of the influenza viruses, and **Paramyxoviridae**, consisting of the parainfluenza viruses, Newcastle disease virus, mumps virus, measles virus and respiratory syncytial viruses. Table 48.1 lists the important differences between orthomyxovirus and paramyxovirus.

Table 48.1 Distinguishing features of orthomyxovirus and paramyxovirus

Property	Orthomyxovirus	Paramyxovirus
Size of virion	80–120 nm	100–300 nm
Shape	Spherical; filaments in fresh isolates	Pleomorphic
Genome	Segmented; eight pieces of RNA	Single linear molecule of RNA

Diameter of nucleocapsid	9 nm	18 nm
Site of synthesis of ribonucleoprotein	Nucleus	Cytoplasm
Genetic reassortment	Common	Absent
Dependent RNA synthesis	Required for multiplication	Not required
Effect of actinomycin D	Inhibits multiplication	Does not inhibit
Antigenic stability	Variable	Stable
Hemolysin	Absent	Present

INFLUENZA

Influenza is an acute infectious disease of the respiratory tract which occurs in sporadic, epidemic and pandemic forms. Influenza also occurs in birds and animals. Birds, particularly aquatic, appear to be the primary reservoir of influenza viruses and natural infection has been identified in several avian species. In birds, it is usually an asymptomatic intestinal infection. The cloaca of healthy wild birds is the best source for the isolation of avian influenza viruses. Type A influenza virus contains animal and human strains while type B consists of exclusively human viruses (natural infection has not been identified in animals or birds). Influenza C contains human and swine viruses. Non-human influenza viruses play an important role in the emergence of pandemic influenza.

Clinical Case

A 60-year-old woman with a history of chronic obstructive airway disease was admitted to the emergency ward with a cough and fever of 39°C. Her fever and cough showed rapid progression and later, she developed dyspnea with respiratory distress. The blood gas analysis was consistent with hypoxia and her chest X-ray showed bilateral ground-glass opacities with areas of consolidation. Her nasopharyngeal aspirate was subjected to PCR, which was positive for influenza virus H1N1. She succumbed to the infection despite supportive care and oseltamivir antiviral therapy.

INFLUENZA VIRUS

Morphology

The influenza virus is usually spherical, with a diameter of 80–120 nm; however, while some strains show pleomorphism. Filamentous forms, up to several micrometres in length and readily visible under the dark-ground

microscope, are frequently seen in freshly isolated strains. The virus core consists of ribonucleoprotein in helical symmetry. The negative-sense single-stranded **RNA genome** is **segmented into eight pieces (13.6 kb overall size)**. Additionally, a viral RNA-dependent **RNA polymerase** is present, which is essential for the transcription of the viral RNA in infected host cells.

The nucleocapsid is surrounded by an **envelope**, which has an inner membrane protein layer and an outer lipid layer. The membrane protein is also known as the matrix or **'M protein'** and is composed of two components, M1 and M2, which are the ion channels. The protein part of the envelope is virus-coded but the lipid layer is derived from the modified host cell membrane during the process of replication by budding. In total, the influenza virus contains nine structural proteins, making up 73% of the composition of the virus. Projecting from the lipid envelope are two types of spikes (**peplomers)**: **hemagglutinin** spikes, which are triangular in cross-section and the mushroom-shaped **neuraminidase** peplomers, which are less numerous (Fig. 48.1).

Fig. 48.1 Influenza virus showing surface protein hemagglutinin (HA), neuraminidase (NA) and the segments of ribonucleoprotein and M2 ion channels (*Source:* Public Health Image

Library, ID 17346 / Douglas Jordan; Dr. Ruben Donis,
Dr. James Stevens Dr. Jerry Tokars, Influenza Division / CDC)

Classification

The classification of influenza viruses into the **three serotypes A, B and C** is based on the antigenic nature of the 'internal' or ribonucleoprotein (RNP) and the matrix (M) protein antigens. These antigens are not cross-reactive amongst the three types.

Influenza virus type A is further divided into subtypes based on the HA and NA glycoproteins.

- 15 HA subtypes are H1–H15
- 9 NA subtypes are N1–N9
- Human isolates belong to H1–H3, H5 and N1, N2 subtypes.

Nomenclature Systems

The standard nomenclature systems include the following information: serotype, host of origin (except for human isolates), geographic origin, strain number and year of isolation, followed by HA and NA subtypes in parenthesis. For a human isolate, it is as follows: A/Hong Kong/01/68 (H3N2) and for other host origin: A/swine/Iowa/15/30 (H1N1) (Table 48.2).

Table 48.2 Classification and nomenclature of influenza virus A subtypes

Old designation	Current classification	Reference strains
A swine	H1N1	A/Swine/Wisconsin/15/30
AO		A/PR/8/34
A1		A/FM/1/4
A2	H2N2	A/Singapore/1/57
A2 (Hong Kong)	H3N2	A/Hong Kong/1/68

Antigen Classification

The internal antigens of the influenza virus can be classified into the following types:

- ***RNP antigen*** This is an internal antigen which is ribonucleoprotein and hence is called the RNP antigen. It can be demonstrated by complement fixation (CFT) and immunoprecipitation tests. It is type-specific and based on its nature, influenza viruses are classified into **types A, B and C**. The RNP antigens of types A, B and C are distinct. The RNP antigen is stable and does not exhibit any significant antigenic variation. Anti-RNP antibody develops after infection but not following killed vaccines.
- ***M protein antigen*** Like the RNP antigen, the M protein is also type-specific and distinct for A, B and C types of influenza viruses. The envelope lipid antigen is host-specific and is determined by the species in which virus replication takes place.
- ***V antigen or the surface antigen*** The term '**viral**' or V antigen was used to describe the surface antigen of the influenza virus. The V antigen is composed of at least two virus-coded proteins, hemagglutinin and neuraminidase. The two proteins have been isolated and purified.

Hemagglutinin is a glycoprotein composed of two polypeptides**, HA 1 and HA 2.** It is responsible for hemagglutination and hemadsorption. It enables the virus to adsorb to mucoprotein receptors on red cells as well as on respiratory epithelial cells. Anti-hemagglutinin antibodies are produced following infection and immunisation. This antibody confers protection by preventing the adsorption of the virus to cells. Hemagglutinin is a strain-specific antigen and is capable of great variation. Fifteen distinct HA subtypes, H1–H15, have been identified in avian influenza viruses but only four of them (H1, H2, H3 and H5) have been found in human isolates so far.

Hemagglutination

Hemagglutination is an important characteristic of influenza viruses. When mixed with a suspension of fowl erythrocytes, the virus is adsorbed onto the mucoprotein receptors on the cell surface. The hemagglutinin peplomers on the viral surface are responsible for this activity. Hemagglutination is followed after a time by the detachment of the virus from the cell surface,

reversing the hemagglutination. This process is known as **elution** and is **caused by neuraminidase** (sialidase) present on the viral surface. The enzyme inactivates *N*-acetylneuraminic acid on the cell receptor.

Virus particles that have eluted from red cells are still capable of agglutinating fresh red cells but red cells that have been acted on by the virus are not susceptible to agglutination by the same strain of the virus. Such red cells may, however, be agglutinated by other myxoviruses. The inability of red cells to be re-agglutinated by the same virus is due to the destruction of the specific cell receptors by initial treatment with the virus.

Receptor gradient Myxoviruses can be arranged in a series in which the treatment of red cells with any one virus removes the receptors for that virus and the preceding viruses but not for the viruses later in the series. For myxoviruses in general, the gradient is mumps, Newcastle disease virus and influenza, in that order.

Hemagglutination takes place within a wide temperature range (0–37°C). Influenza viruses vary in their ability to agglutinate red cells of different species. In general, types A and B agglutinate erythrocytes of fowls, humans, guinea pigs and other species. Influenza virus type C agglutinates the red cells of fowls only, at 4°C.

Neuraminidase is a glycoprotein enzyme which destroys cell receptors by hydrolytic cleavage. The anti-neuraminidase antibody is formed following infection and immunisation. It is not as effective in protection as the anti-hemagglutinin antibody. It does not prevent the adsorption of the virus onto cells but can inhibit the release and spread of progeny virions and may thus contribute to limiting the infection. It is a strain-specific antigen and exhibits variation. **Nine different subtypes** have been identified (N1–N9). Of these, only N1 and N2 have been recovered from humans.

Neuraminidases are also present in bacteria and in the cells of higher organisms. Culture filtrates of *V. cholerae* are rich in neuraminidase activity and red cells pre-treated with them are resistant to hemagglutination by influenza viruses. The culture filtrate was therefore called the receptor-destroying enzyme (RDE) of *V. cholerae*.

Antigenic Variation

A unique feature of the influenza virus is its ability to undergo

antigenic variation. This has an impact on the epidemiology of the disease. Antigenic variability is highest in influenza virus type A, is lesser in type B and has not been demonstrated in type C.

The internal RNP antigen and M protein antigen are stable but both **surface antigens undergo independent antigenic variations** which may be of two types:

1. ***Antigenic drift*** The **gradual** sequential change in antigenic structure occurring regularly at **frequent intervals** is known as **antigenic drift**. Here, the new antigens, though different from the previous antigens, are still related to them, so they react with antisera to the predecessor virus strains to varying degrees. Antigenic **drift is due to mutation and selection**, the process being influenced by the presence of antibodies to the predecessor strains in the host population. Antigenic drift accounts for the periodic epidemics of influenza.
2. ***Antigenic shift*** Unlike antigenic drift, this is an abrupt, drastic and discontinuous variation in the antigenic structure, resulting in a **novel virus** strain, unrelated antigenically to the predecessor strains. Such changes may involve hemagglutinin, neuraminidase or both. Antibodies to the predecessor viruses do not neutralise the new variant and spread widely in the population, causing major **epidemics** or **pandemics.** The changes involved in antigenic shift are too extensive to be accounted for by mutation.

Resistance

The virus is inactivated by heating at 50°C for 30 minutes. It remains viable at 0–4°C for about a week. It can be preserved for years at –70°C or by freeze-drying. The virus survives slow drying and may remain viable on fomites for about two weeks. Ether, formaldehyde, phenol, salts of heavy metals and many other chemical disinfectants destroy infectivity. Iodine is particularly effective.

Hemagglutinating, enzymic and complement-fixing activities of the virus are more stable than infectivity.

Pathogenesis

The route of entry is the respiratory tract. Small doses by aerosols can cause infection. Larger doses are required when infection is by intranasal instillation. The viral neuraminidase facilitates infection by reducing the viscosity of the mucus film lining the respiratory tract and exposing the cell surface receptors for virus adsorption. At this stage, the virus may get inactivated by mucosal IgA or other non-specific inhibitors. The viruses that bypass this mechanism affect the ciliated cells. **The ciliated cells of the respiratory tract are the main sites of viral infection**. These cells are damaged and shed by the viral neuraminidase, laying bare the basal cells in the trachea and bronchi. This renders the respiratory tract highly vulnerable to bacterial invasion. Viral pneumonia is associated with hyperemia and thickening of the alveolar walls, interstitial infiltration with leucocytes, capillary thrombosis and leucocytic exudation. In some cases, a hyaline membrane is formed, occupying the alveolar ducts and alveoli. In the late stages, there is
infiltration with macrophages which engulf and remove desquamated alveolar cells. The disease is confined to the respiratory tract.

Clinical Features

The **incubation period** is 1–3 days. The disease varies in severity from mild coryza to fulminating and rapidly fatal pneumonia. Most infections are subclinical.

The **clinical onset** is abrupt, with fever, headache and generalised myalgia. Respiratory symptoms are prominent with sore throat and cough. Severe prostration is common. Abdominal pain and vomiting may occur, especially in type B infection in children, which may even present as an acute abdominal emergency. The uncomplicated disease resolves within about seven days. Very young and elderly individuals, pregnant women and immunocompromised individuals are at a greater risk of infection.

Complications The most significant complication is **pneumonia,** which is mainly due to bacterial superinfection or, rarely, caused by the virus itself (*Clinical case*). **Cardiac complications**, such as congestive failure or myocarditis and neurological involvement, such as encephalitis, may occur rarely.

Influenza, particularly infection with type B, has been associated with **Reye**

syndrome (encephalopathy, fatty liver changes with raised transaminase). It especially affects young children. Type B infections may sometimes cause gastrointestinal symptoms (gastric flu).

High-risk groups Elderly individuals, diabetics, persons with kidney and liver disease, those with cardiac disease, pregnant women, immunocompromised individuals and cancer patients are at a greater risk of infection.

Laboratory Diagnosis

1. **Specimen collection** Throat garglings, nasal wash or throat swabs (alginate swabs) are collected using virus transport media or suitable buffered salt solution. If the specimen is not processed immediately, it should be stored at 4°C, or if the delay is long, at –70°C.

2. **Demonstration of the virus antigen** Rapid diagnosis of influenza may be made by the demonstration of the virus antigen on the surface of the nasopharyngeal cells by immunofluorescence.

3. **Isolation of the virus** Virus isolation is obtained readily during the first two or three days of the illness but less often in the later stages. The virus grows in primary monkey kidney cell cultures, as well as in some continuous cell lines. Cytopathic effects are not prominent and **virus growth is detected by hemadsorption or demonstration of hemagglutinin** in the culture fluid.

Isolation may also be made in eggs in the **amniotic cavity of chick embryos**. After a few egg passages, the virus grows well in the allantoic cavity also, except for the type C virus which does not generally grow in the allantoic cavity. Hemagglutination provides a convenient method for the detection and titration of the influenza virus in egg and other culture fluids. The highest dilution of the virus suspension that produces agglutination of a fixed quantity of cells is known as its hemagglutination (HA) titre. Hemagglutination is used to titrate inactivated influenza virus for standardisation of killed influenza virus vaccines.

The virus can be ‘adapted’ by serial intranasal passage in mice to produce fatal pulmonary infection. Neurotropic mutants have been isolated, which regularly produce fatal encephalitis after intracerebral inoculation in mice.

Subtype identification of some strains is made by hemagglutination

inhibition test or by demonstrating virus antigen in infected cell cultures by **immunofluorescence**.

- **Hemagglutination and elution** can be used for purifying and concentrating influenza viruses along with subtype identification.
- Virus growth can be identified by hemadsorption with human O group, fowl or guinea pig erythrocytes, on tissue culture cells in which the virus is multiplying as they contain the hemagglutinins. Red cells are adsorbed onto the surface of such cells; this is known as **hemadsorption**.
- Shell vial cultures can be done and the virus identified in 1–4 days by monoclonal antibodies.
- RT-PCR can be done to rapidly identify the culture products.

4. **Serology** Several antibodies are produced against hemagglutinin, matrix, neuraminidase and nucleoprotein. Serodiagnosis is based on hemagglutination inhibition tests. It is essential to examine paired sera in parallel, to demonstrate a rise in titre of antibodies.

Hemagglutination inhibition (HAI) offers a convenient and sensitive method for the detection and quantitation of the antibody to the virus and the serological diagnosis of influenza. However, it has some disadvantages. As the anti-hemagglutinin antibodies are subtype-specific, it is necessary to use as antigens to the strain currently causing infection. The sera, suitably treated for the removal of non-specific inhibitors,
are diluted serially in hemagglutination plates and the influenza virus suspension containing 4 HA units is added to each cup. Fowl red cells are then added. The highest dilution of serum that inhibits hemagglutination is its
HAI titre.

5. **PCR-based diagnosis** With specific primers to detect the RNA by reverse transcriptase PCR (RT-PCR), the subtypes can also be detected by a multiplex PCR assay to identify the virus in a clinical specimen. This technique can also identify several other respiratory viruses from the clinical specimen. **This is now the preferred method due to its sensitivity, rapidity and ease of performance.** The results are available in real time (<1 day), making isolation faster and allowing containment

practices to be put in place rapidly.

Immunity

An attack of influenza confers **effective protection for one or two years.** The apparent short duration of immunity is due to the antigenic variation that the virus undergoes frequently. It is the local concentration of anti-hemagglutinin and, to a smaller extent, of anti-neuraminidase antibodies (mainly IgA) in the respiratory tract that is more relevant in protection.

'Original antigenic sin' When an individual experiences repeated infections with different antigenic variants of influenza virus type A, he responds by forming antibodies not only against each infecting strain but also against the strain that he first came into contact with. The dominant antibody response will be against the strain that caused the earliest infection. This is known as the doctrine of 'original antigenic sin'.

Influenza virus infection induces cell-mediated immunity also but its role in protection has not been clarified.

Epidemiology

Influenza occurs sporadically as epidemics or in pandemic form. The source of infection is an infected individual. Influenza virus type C is **endemic** throughout the world and causes very mild or unapparent infections. Type B strains cause sporadic as well as epidemic influenza, while type A strains can cause pandemics as well.

Epidemic influenza is significant in temperate regions during the winter months, causing considerable mortality in the aged and in those with cardiopulmonary diseases. In the tropics, it tends to occur frequently in the monsoon season.

Pandemics make influenza an important and challenging disease. Worldwide surveillance is maintained on influenza, under the auspices of the WHO. Influenza pandemics have been recorded at irregular intervals from 1173. The H1N1 virus, which emerged in China and Russia in 1977, has spread across most parts of the world. The H3N2 virus **currently causes human influenza**.

Ability to cause epidemics and pandemics

The reason the virus is able to cause epidemics and pandemics is its ability to **undergo antigenic variation**.

- **Antigenic drift**, resulting from mutation and selection, is responsible for the epidemics.
- **Pandemics are caused by a virus strain that has undergone antigenic shift**. The variation in such instances is so marked and involves so many different polypeptides simultaneously that mutation cannot explain it. It is now held that **pandemic strains originate from some animal or avian reservoir**, either spreading to humans directly by host range mutation, or as a result of recombination between human and non-human strains.
- Hybrids can be produced by growing human and non-human strains together in eggs. Recombinants can also be obtained from experimental animals exposed to mixed infection (e.g., the 1957 Asian virus and the 1968 Hong Kong virus were recombinant hybrids).

Avian influenza

The mere appearance of a new or hybrid strain may not lead to a pandemic. The new strain should be capable of spreading rapidly among people. In fact, there have been several instances when new hybrids have been detected, which failed to spread. The swine flu virus H1N1 caused a localised outbreak in a military camp in New Jersey, USA, in 1976, leading to much anxiety and panic vaccination but it did not spread. In 1997, the threat of a new pandemic in Hong Kong due to chicken flu caused by H5N1 influenza virus resulted in 18 confirmed human cases; however, it did not get transmitted from person to person. Immediate containment measures stopped the danger before the strain developed person-to-person transmissibility. This incident indicated the value of influenza surveillance and the potential danger from avian strains. Despite several poultry outbreaks, India has not reported a single human H5N1 case. The highly pathogenic avian influenza (bird flu), caused by the H5N2 strain was reported from some countries in 1914. To combat the outbreak, millions of birds were culled in several countries with great economic loss.

It is now known that wild aquatic birds (Fig. 48.2) carry the full repertoire of the genes of all influenza strains, including old human pandemic strains, and that the viruses do not cause any disease in them or undergo any mutational changes. Birds shed the viruses abundantly in feces, which contaminate lakes and ponds. Recombination may take place in pigs and such **hybrid strains may lead to human infection with potential pandemic spread**. **The reappearance of old strains, like the H1N1 in 1977, may have been from an avian reservoir of strains**.

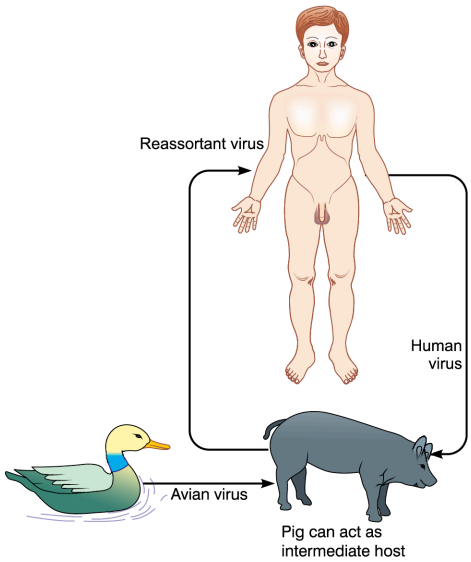


Fig. 48.2 Transmission cycle of avian influenza virus: Human → pig ← aquatic bird (waterfowl)

A unique feature of influenza epidemiology was that once an antigenic variant emerged, it completely displaced the pre-existing strain. Thus, when A1 (H1N1) strains arose in 1946–47, they became the only viruses causing human disease and the previous A0 (H0N1) strains disappeared completely. However, this rule has not been observed in recent years. Even after the re-emergence and wide dissemination of the H1N1 strain in 1977, the A2 Hong Kong H3N2 strains continue to be prevalent. The reason for this co-existence is not known.

There is an orderly recycling of the virus subtypes, at least, with regard to their hemagglutinin (H) antigen. Seroepidemiological (seroarcheological) studies indicate that the severe pandemics are generally followed by moderate ones. The pandemic in 1957 with the H2N2 (Asian) subtype was followed in 1968 by the H3N2 (Hong Kong) virus, leading to a moderate pandemic. The year 1977 saw the reappearance of the H1N1 virus. Thus,

the sequence of variation in the H antigen has been
H2 → H3 → H1 → H2 → H3 → H1 from 1889 to the present time. From 1977, both H3N2 and H1N1 viruses have been circulating together.

Swine influenza I

In March 2009, a new H1N1 virus was detected, which was a reassortant between previously circulating swine virus and a Eurasian swine virus and was also called swine-origin influenza. Human cases of influenza A pandemic (H1N1) have been reported globally since 2009 with several thousand deaths. This virus has now become the seasonal influenza in various parts of the world, including India, and is expected to continue so for many years. Annually, several cases are admitted to the hospitals during the colder seasons.

Current status of influenza virus infection in India

The number of reported cases of seasonal influenza (H1N1) in 2019 (till 21 April 2019) is 24,018 with 880 deaths (based on the reports received from the states/UTs to the Central Surveillance Unit, Integrated Disease Surveillance Programme, NCDC, Delhi. (https://ncdc.gov.in/)

Immunoprophylaxis

Influenza vaccines have been in use for many decades and are the mainstay in the prevention of influenza.

The main **difficulty in the immunoprophylaxis of influenza is the frequent change in the antigenic makeup of the virus**. In cold countries, where it is necessary to protect old persons and other high-risk individuals, the practice is to immunise them with a **vaccine containing the latest strains of type A and B viruses**.

The most important indication for immunoprophylaxis is when a pandemic is threatened by a new virus.

The vaccines are of **two types:**

1. ***Inactivated influenza vaccines (IIV)*** The original vaccines consisted of viruses grown in the allantoic cavity of eggs, partially purified, and inactivated with formalin. Due to the presence of egg protein, this vaccine may cause reactions in allergic individuals. The whole virus vaccine induces fever and local pain.

- **Subunit vaccines** have been introduced to minimise toxic reactions. The purified virus is disrupted by treatment with detergents so that the vaccine contains the immunogenic hemagglutinin.
- **Recombinant vaccine** is a vaccine with antigens from a new variant. A recombinant possesses the growth characters of old established strains and carries the surface antigens of the new variant. Moreover, most fresh isolates do not grow well in eggs till they are passaged serially. The recombinant will grow well in eggs, facilitating vaccine manufacture.
- **Killed vaccines** induce the formation of circulating antibodies and do not lead to any local protection in the respiratory tract. The level of antibodies on the respiratory mucosa is only a fraction of the serum level.

Indications for IIV IIV is approved for use in persons 6 months and older, including pregnant women and persons with chronic medical conditions and other high-risk groups.

2. ***Live attenuated (cold-adapted) influenza vaccine (LAIV)*** To enable specific local immunisation, live vaccines have been used. The live attenuated vaccine is a temperature-sensitive mutant. **Ts mutants** can be readily isolated, which are able to grow at the lower temperature of the nasopharyngeal mucosa (32–34°C) but not in the lungs at 37°C. Such **ts** mutants are avirulent. Recombinant live vaccines may be obtained by hybridisation between the ts mutants of established strains and a new antigenic variant.

Indications for live influenza vaccine LAIV is approved for use only in persons aged 2–49 years who do not have underlying medical conditions. The vaccine should not be administered to pregnant women.

The World Health Organization recommends vaccination of high-risk groups with seasonal influenza vaccine. Individuals with increased risk of complications due to influenza virus include elderly individuals >65 years of age, children with asthma, immunocompromised persons, those with chronic obstructive pulmonary disease, bronchial

asthma, heart disease, liver disease, kidney disease, blood disorders, diabetes, and cancer, as well as persons who might transmit to other high-risk groups (health care providers attending to persons with influenza).

Seasonal influenza caused by HINI, H3N2 and influenza B affects the elderly (>65 years) and young children, who are at a higher risk. The Ministry of Health and Family Welfare, Directorate General of Health Services (National Centre for Disease Control) has recommended '**Seasonal Influenza: Guidelines'** for vaccination with quadrivalent influenza vaccine composed of:

- A/Michigan/45/2015 (H1N1)pdm09-like virus
- A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus
- B/Phuket/3073/2013-like virus (B/Yamagata/ 16/88 lineage)
- B/Brisbane/60/2008-like virus (Victoria lineage)

Chemoprophylaxis has been reported to be successful with the antiviral drugs amantadine and rimantadine which block the viral M2 protein, which functions as an ion channel. These act only with the type A virus and not with the type B, which lacks the M2 components.

Treatment

There are four approved antiviral drugs that are prescribed within the first day or two of symptoms to reduce the severity of symptoms and possibly, the risk of complications. These include oseltamivir (Tamiflu), zanamivir (Relenza), peramivir (Rapivab) and baloxavir (Xofluza). Zanamivir and oseltamivir, which are designed to block viral neuraminidase, have been found effective in the treatment and prevention of influenza, when administered as a nasal spray. The virus can develop resistance to these drugs.

Amantadine and rimantadine are useful in the treatment of influenza. They reduce the average duration of the disease and cause symptomatic improvement, though virus shedding and antibody response are not affected. Resistance to these drugs develops rapidly.

Key points

- The Orthomyxoviridae family comprises enveloped, helical RNA viruses, 80–120 nm in diameter.
- The single-stranded RNA genome is segmented into eight pieces, each piece representing a gene. Genetic reassortment of the various genes of influenza A is a common natural event.
- The virus envelope consists of hemagglutinins and neuraminidase, HA and NA.
- The influenza A virus is the major cause of the epidemic and pandemic forms of influenza.
- Major antigenic changes (antigenic shift) and continuous minor variations (antigenic drift) are features of this virus. Genes for 15 hemagglutinins and 9 neuraminidases are known. The virus may be isolated in fertile hens' eggs and in monkey kidney primary cells.
- The influenza B and C types do not have subtypes.
- Influenza is an acute upper respiratory disease characterised by fever, headache, chills, malaise, myalgia, anorexia, sore throat, respiratory symptoms of non-productive cough, rhinorrhea, sneezing and nasal obstruction. Droplets containing the virus are transmitted by aerosol from person to person.
- Antibody is protective when it is directed at the hemagglutinin (the adhesin of influenza viruses) by neutralising the virus, which prevents the virus from binding to host cells.
- For diagnosis, the virus can be grown in tissue culture cells or chick embryos; fluorescent antibody can be used to detect viral antigens in nasopharyngeal cells; serology by HAI or CFT can be used if done in a paired serum sample.
- Inactivated vaccines are developed every year in developed countries to keep pace with the yearly strains of influenza viruses A and B. Rimantadine or amantadine can be given to treat or prevent type A infection only. Neuraminidase inhibitors are new agents effective against both influenza A and B.

Questions

Essays

1. Describe the classification and nomenclature system of the influenza virus.
2. Describe the epidemiology of influenza virus infection.

Short Answers

1. Antigenic shift and antigenic drift
2. Influenza pandemics
3. Antiviral agents for influenza
4. Avian influenza
5. Hemagglutinin of the influenza virus
6. Neuraminidase of the influenza virus
7. Influenza vaccines
8. Structure of the influenza virus

CHAPTER 49

Paramyxovirus, Pneumovirus and Rubella

Chapter navigation

- Parainfluenza
- Measles
- Mumps
- Hendra
- Nipah
- Pneumovirus
  - Respiratory syncytial virus
  - Metapneumovirus
- Rubella
- Other respiratory viruses

INTRODUCTION

The family Paramyxoviridae contains important pathogens of infants and children, responsible for a major part of acute respiratory infections (respiratory syncytial virus and parainfluenza viruses) and also for two of the most contagious diseases of childhood (measles and mumps). Though much less common, infections may also occur in adults.

Paramyxoviruses resemble orthomyxoviruses in morphology but are larger and more pleomorphic (Table 49.1). They are roughly spherical in shape and range in size from 100 nm to 300 nm, sometimes with long filaments and giant forms of up to 800 nm. The helical nucleocapsid has a diameter of 18 nm (except in the pneumovirus, where it is 13 nm). The genome is a molecule of linear **single-stranded RNA.**

Unlike orthomyxoviruses, in which the segmented nature of the genome facilitates the genomic reassortment and antigenic variation so typical of influenza viruses, the **paramyxoviruses with their unsegmented genome**

do not undergo genetic recombination or antigenic variation. Hence all paramyxoviruses are **antigenically stable.**

Table 49.1 Important characteristics of Paramyxoviridae

Properties	Paramyxovirinae				Pneumovirinae	
	Respirovirus	Rubulavirus	Morbillivirus	Henipavirus	Pneumovirus	Metapneumovirus
	Parainfluenza 1–3	Mumps Parainfluenza 2–4	Measles	Nipah Hendra	Resp. syncytial virus	Human metapneumovirus
Diameter of nucleocapsid	18 nm				13 nm	
Fusion (F) protein	Fusion protein is present in all of them					
Hemagglutinin/ Hemadsorption	Possess HA and exhibit hemadsorption on specific RBCs			Do not have HA and do not exhibit hemadsorption		
Neuraminidase (NA)	Neuraminidase on the surface		NA is absent	Do not possess NA on the surface		
Intracellular inclusions in cytoplasm (C) / nucleus (N)	Intracytoplasmic inclusion bodies		Intranuclear and Intracytoplasmic	Intracytoplasmic inclusion bodies		Not known

ANTIGENIC STRUCTURE OF PARAMYXOVIRUSES

The **nucleocapsid** (Fig. 49.1) is surrounded by a **lipid envelope** which has the **matrix (M)** protein at its base and two types of transmembrane glycoprotein spikes at the surface. The longer spike is **hemagglutinin (H)**, which may also possess neuraminidase (N) activity and is hence known as the H or **HN protein.** It is responsible for the adsorption of the virus to the host cell surface.

The second spike is the **F (fusion) protein**, responsible for the fusion of the viral envelope with the plasma membrane of the host cell, which is the essential early step of infection. It also brings about cell-to-cell fusion, forming large giant cells or **syncytia**, which are characteristic of paramyxovirus infections. The F protein also mediates the hemolytic activity of paramyxoviruses.

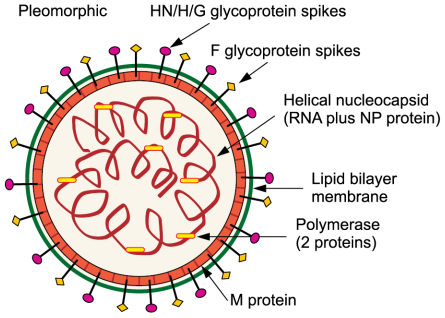


Fig. 49.1 Mumps virus

CLASSIFICATION OF PARAMYXOVIRUSES

The family Paramyxoviridae is divided into two subfamilies: (i) Paramyxovirinae and (ii) Pneumovirinae. All the genera are monotypic (one serotype) and are antigenically stable.

i) Paramyxovirinae

- Respirovirus–Parainfluenza 1 and 3
- Rubulavirus–Parainfluenza 2, 4a, 4b and mumps
- Morbillivirus–Measles
- Henipavirus–Nipah and Hendra

ii) Pneumovirinae

- Pneumovirus–Respiratory syncytial virus
- Metapneumovirus–Human metapneumovirus

Some animal viruses are related to human paramyxovirus:

- Sendai virus of mice—related to subtype of human parainfluenza type I virus
- Simian parainfluenza virus 5 (SPIV5)—the same as canine parainfluenza type 2 and contaminates monkey kidney cell line
- Shipping fever virus of cattle—a subtype of parainfluenza type 3

PARAINFLUENZA VIRUS

Parainfluenza viral infection is confined to the respiratory tract, unlike mumps which is a systemic disease, with the virus disseminating through blood and multiplying in various organs and tissues. The causative viruses are responsible for about 10 per cent of respiratory infections in children needing hospitalisation.

All human parainfluenza types are strongly correlated with specific clinical syndromes, ages and times of the year. PI types 1 and 2 are the pathogens most commonly associated with **croup,** a serious clinical disease**.**

PI type 3 is the pathogen most commonly associated with bronchitis, bronchiolitis and pneumonia in infants and young children. Type 4 causes minor respiratory illnesses.

In adults, parainfluenza viruses cause milder respiratory infections in which sore throat and hoarseness of the voice are common. Rarely, they cause parotitis. Otitis media is a common complication in immunocompromised individuals.

Epidemiology

Parainfluenza virus type 3 infection is often experienced in the first year of life with about 50 per cent of infants being seropositive by 12 months of age. Types 1 and 2 cause disease mainly in preschool children. Type 3 infection is more endemic than types 1 and 2 which tend to occur as epidemics. Immunocompromised individuals have increased susceptibility to the parainfluenza virus infections.

First infections are more serious than re-infections, which are not infrequent. With the type 4 virus, even first infections are very mild. Infected children shed the virus in respiratory secretions for about a week. Spread is by air or through fingers. Nosocomial spread is not uncommon. **No vaccine is available.**

Laboratory Diagnosis

1. **Specimen** Throat and nasal swabs are collected for laboratory investigations.

2. **Virus isolation** Throat and nasal swabs are inoculated in primary monkey kidney cell cultures or continuous monkey kidney cell lines (LLC–MK2) with trypsin.

- Cytopathic changes are not readily apparent, except with the type 2 virus. Isolation may take ten days or more.
- Virus growth is detected by hemadsorption. Typing is by immunofluorescence, hemadsorption inhibition or hemagglutination inhibition.

3. **Serology** Serological diagnosis is hampered by wide antigenic cross-

reactions. Paired sera can be tested by neutralisation, ELISA, HAI or CF (complement fixation) for rise in the titre of antibodies.

4. **PCR** Molecular diagnosis using reverse transcriptase PCR is gaining acceptance as a more specific and rapid technique.

MUMPS VIRUS

The mumps virus causes acute parotitis (mumps), an infection commonly affecting children. It is characterised by **non-suppurative enlargement of the parotid glands**.

> The mumps virus possesses both HA, NA and F proteins. It agglutinates the erythrocytes of fowls, guinea pigs, humans and many other species. Hemagglutination is followed by hemolysis and elution at 37°C. As in influenza viruses, two complement-fixing antigens can be recognised—the soluble (S) antigen and the viral (V) antigen.

Resistance

The mumps virus is labile, being rapidly inactivated at room temperature or by exposure to formaldehyde, ether or ultraviolet light. It can be preserved at –70°C or by lyophilisation.

Clinical Features

Route Infection is acquired by inhalation.

Incubation period It is about 12–25 days.

Spread The virus replicates in the nasal and upper respiratory tract and cervical lymph nodes and is disseminated through the bloodstream to various organs. The virus is shed from the saliva three days before to about 9 days after salivary gland swelling.

Inapparent infection Almost one-third of infections are subclinical including the majority in children less than 2 years of age.

Parotid swelling is usually the first sign of illness, though it may sometimes be preceded by prodromal malaise. Parotid swelling is unilateral to start with but may become bilateral (Fig. 49.2). It is accompanied by fever, local pain and tenderness but the skin over the gland is not warm or erythematous. Parotitis usually resolves within a week. However, involvement of extraparotid sites may be more serious and may sometimes

occur even in the absence of parotitis.

Fig. 49.2 Parotid gland swelling in a child with mumps (*Source:* Public Health Image Library, ID 3186 / Heinz F. Eichenwald, MD / CDC)

Complications

- **Epididymo-orchitis** is a complication seen in about a third of postpubertal male patients. The testis becomes swollen and acutely painful, with accompanying fever and chills. **Orchitis** is usually unilateral but when it is bilateral and followed by testicular atrophy, sterility or low sperm counts may result.
- **Meningitis —** the central nervous system is involved in about 60 per cent of cases, as indicated by pleocytosis in the CSF, but only about 10 per cent show symptoms of meningitis**.** Mumps has been reported to cause about 10–15 per cent of cases of **aseptic meningitis.** It usually resolves without sequelae.
- **Meningoencephalitis may also occur,** but like meningitis, it usually resolves without sequelae; deafness may sometimes result. Meningoencephalitis may present 5–7 days after infection of the parotids but about half the patients will not have symptoms of parotitis presenting as **atypical mumps**. The virus can be grown readily from the CSF in the early phase of meningitis.
- Other less common complications are arthritis, oophoritis, nephritis, pancreatitis, thyroiditis and myocarditis.

Epidemiology

Mumps is endemic worldwide but has become less common due to immunisation. It often occurs as epidemics in children between 5 and 15 years of age and also in young people living in groups such as in army

camps. Household spread is common. Humans are the only natural hosts. The source of infection is a patient in the late incubation or early clinical stage of the illness. No human carriers or animal reservoirs exist.

Infection is transmitted by direct contact, airborne droplets or fomites contaminated with saliva, and also possibly, urine. The virus is detectable in saliva for about a week before and a week or two after onset of parotitis. However, peak infectivity is about a day or two before parotitis becomes evident and subsides rapidly thereafter. The virus is also shed in urine for up to two weeks after the clinical symptoms begin, though its role in the transmission of infection is not clear.

One attack of mumps confers lasting immunity so that second attacks do not occur.

Immunity

Infection leads to antibody response against both the internal (S) and surface (V) antigens. Antibodies to the S antigen appear early, within 3–7 days of the onset of symptoms, but disappear after about six months. The anti-hemagglutinin antibody correlates well with immunity to infection. Even subclinical infections lead to hemagglutinin antibody and resistance to infection. As antibodies are widespread in the population, passive immunity protects newborns. Mumps is, therefore, very rare before six months of age.

Laboratory Diagnosis

A classic case of mumps does not need laboratory confirmation but it may be essential in atypical infection and where meningitis or other systemic involvement is the sole manifestation. Diagnosis may be established by virus isolation and serological tests.

1. **Specimen** The virus may be isolated from saliva (within 4–5 days), urine (up to two weeks) or CSF (8–9 days after the onset of the illness).
2. **Virus isolation**

- Cell cultures are better suited for isolation—primary monkey kidney being the preferred cell. Human amnion and HeLa cells also support the growth of the mumps virus. Cytopathic effect is slow and consists of syncytium formation and the presence of

acidophilic cytoplasmic inclusions. Growth is best identified by hemadsorption. Virus growth can be detected by hemadsorption and identified by hemadsorption inhibition using specific antiserum. Rapid results can be obtained by immunofluorescence of infected cell cultures. This may become positive as early as 2–3 days after inoculation.

- The virus can also be grown in chick embryos—in the amniotic cavity for primary isolation and the allantoic cavity after adaptation. Embryonated (fertilised) eggs are inoculated with the specimen and incubated for 6–8 days at 35°C. The amniotic fluid is then harvested and tested for agglutinins, the presence of which indicates the growth of the virus. The virus can be identified by hemagglutination inhibition using specific antisera. Egg inoculation is less sensitive than cell cultures for isolation.

3. **Antigen detection** Direct antigen detection by IFA is helpful in early diagnosis.

4. **Serology** Serological diagnosis depends on the demonstration of a rise in the titre of antibodies in paired serum samples. IgM-ELISA is useful in this respect because cross-reacting antibodies are IgG and do not interfere with IgM-ELISA.

The CF and HAI tests are commonly used but cross-reactions with parainfluenza viruses cause problems. A positive CF test for antibody to the S antigen in the acute-phase serum is presumptive evidence of current infection.

5. **PCR** Molecular diagnosis using **reverse transcriptase PCR** is more rapid and sensitive.

Prophylaxis

Vaccination An effective live virus vaccine is available against mumps. The **Jeryl–Lynn strain** of the mumps virus, attenuated by passage in eggs and grown in chick embryo fibroblast culture, is used as the vaccine. **It is recommended for use only after one year of age as maternal antibodies may interfere with the multiplication of the vaccine virus if given earlier**.

Vaccination is contraindicated in pregnancy, immunodeficiency and hypersensitivity to neomycin or egg protein. The vaccine is given as a single subcutaneous injection, alone or in combination with the measles and rubella vaccines (**MMR vaccine**). Children should get two doses of **MMR vaccine**, starting with the first dose at 12 to 15 months of age, and the second dose between 4 to 6 years of age. It provides effective protection for at least ten years. The vaccine may not prevent the disease if given after exposure to the infection but there are no contraindications for its use in this situation. A **quadrivalent MMRV** (measles-mumps-rubella-varicella) combination vaccine has recently been launched. This vaccine is highly immunogenic, with seroconversion rates against all antigens.

NEWCASTLE DISEASE VIRUS (NDV)

The Newcastle disease virus (avian paramyxovirus type 1) is a natural pathogen of poultry. Human infection with NDV is confined to self-limited conjunctivitis in poultry workers and others in contact with infected birds.

In India, it is known as the Ranikhet virus. Control measures consist of vaccination, and slaughter of infected birds. Other types of avian paramyxoviruses cause inapparent infection in many species of birds.

PNEUMOVIRUS

RESPIRATORY SYNCYTIAL VIRUS (RSV)

The virus causes cell fusion and the formation of **multinucleated syncytia in cell cultures**, and so, is named respiratory syncytial virus (RSV). It is now recognised as the most common cause of lower respiratory tract infections in infants, particularly in the first few months of life. RSV is pleomorphic and has a size range of 150–300 nm. The viral envelope has two glycoproteins—the G protein by which the virus attaches to cell surfaces, and the fusion (F) protein which brings about fusion between viral and host cell membranes. The F protein is also responsible for cell-to-cell fusion, which leads to the characteristic syncytial cytopathic changes in RSV infection.

RSV does not possess hemagglutinin activity, nor does it possess neuraminidase or hemolytic properties, and thus differs from other

paramyxoviruses. Another difference is that its nucleocapsid diameter (13 nm) is less than that of other paramyxoviruses (18 nm).

It is propagated on heteroploid human cell cultures such as HeLa and HEp-2. It is highly labile and is inactivated rapidly at room temperature. It can be preserved by lyophilisation. It is antigenically stable and **only one antigenic type e**xists. However, studies using monoclonal antibodies have identified **two subtypes, A and B.**

Clinical Features

Most RSV infections are symptomatic. Infection causes a broad range of respiratory illnesses.

- In infants, the disease may begin as febrile rhinorrhea, with cough and wheezing, progressing in 25–40 per cent to lower respiratory involvement, including tracheobronchitis, bronchiolitis and pneumonia. In about one per cent, the illness is serious enough to require hospitalisation. Most patients recover in 1–2 weeks but those with immunodeficiency or cardiac defects may have protracted illness and high death rates.
- In young children, RSV is an important cause of **otitis media**.
- In adults, RSV infection may present as a febrile common cold.
- In the elderly, it can cause pneumonia.

Epidemiology

RSV is global in distribution. It causes annual epidemics in the temperate regions during winter and in the tropics during the rainy season. Outbreaks are common in children's wards, nurseries and day-care centres. Infection is most **common in children between six weeks and six months of age**, with the peak at 2–3 months. Newborns are believed to be protected by high levels of maternal antibodies.

The virus is transmitted by close contact and through contaminated fingers and fomites. Coarse droplets of respiratory secretions discharged during coughing and sneezing are more efficient in spreading the virus than fine aerosols. The incubation period is 4–6 days. Virus shedding may persist for 1–3 weeks, though children with defective cell-mediated immunity may

continue to shed the virus for months.

Re-infection with the virus is not uncommon but the disease so produced is milder than in primary infection. The role of the antibody in protection against the infection is not clear. **Secretory IgA is considered more important than circulating IgG in protection**. Cell-mediated immunity appears more important than humoral antibodies in recovery from infection. RSV does not induce high levels of interferon production.

Laboratory Diagnosis

1. **Specimen** Nasopharyngeal swabs or nasal washings are collected for investigations.
2. **Virus isolation** Samples are inoculated onto cell cultures (HeLa or HEp-2) immediately after collection. Unlike most viruses, freezing of clinical samples may destroy the virus. In cultured cells, RSV causes characteristic giant cell and syncytial formation, but cytopathic effects take about 10 days to appear. Earlier detection of viral growth in cells is possible by immunofluorescence tests. Rapid diagnosis of RSV infection can be made by the immunofluorescence test on smears of nasopharyngeal swabs.
3. **Serology** Serological diagnosis is by demonstration of rising antibody titres in paired serum samples by ELISA, CF, neutralisation or immunofluorescence tests.
4. **PCR** Molecular diagnosis by reverse transcriptase PCR is sensitive and rapid.

Prophylaxis

No effective vaccine is available.

Treatment

Management is primarily by supportive care. Administration of ribavirin by continuous aerosol has been found beneficial in hospitalised patients, decreasing the duration of illness and of virus shedding.

MORBILLIVIRUS

MEASLES (RUBEOLA) VIRUS

The virus has the general morphology of paramyxoviruses. It is a roughly spherical but often pleomorphic particle, 120–250 nm in diameter. The tightly coiled helical nucleocapsid is surrounded by the lipoprotein envelope carrying on its surface hemagglutinin (H) spikes (Fig. 49.3). The envelope also has the F protein which mediates cell fusion and hemolytic activities. The measles virus agglutinates monkey erythrocytes but there is no because the virus does not possess neuraminidase activity.

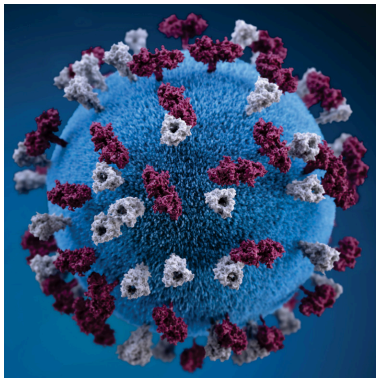


Fig. 49.3 Measles virus with H spikes and F protein (*Source:* Public Health Image Library, ID 21074 / Allison M. Maiuri, MPH, CHES / CDC)

Clinical Case

A 5-year-old boy was brought to a primary health centre with complaints of fever, cough, running nose and conjunctivitis. On examination of his throat, a white spot was noticed on the buccal mucosa near the molars. He was given antipyretics and advised rest. He returned two days later with maculopapular rash on the face, chest and back. He tested positive for measles virus IgM antibody by ELISA. The patient improved with supportive care and aspirin.

The measles virus grows well on human or monkey kidney and human amnion cultures which are the preferred cells for primary isolation. Isolates can be adapted for growth on continuous cell lines (HeLa and Vero) and in the amniotic sac of hen's eggs. Cytopathic effects consist of **multinucleate syncytium formation** with numerous acidophilic nuclear and cytoplasmic inclusions. Multinucleate giant cells (**Warthin–Finkeldey cells**) are also found in the lymphoid tissues of patients.

The virus is labile and readily inactivated by heat, ultraviolet light, ether and formaldehyde. It can be stabilised by molar $MgSO_4$ so that it resists heating at 50°C for one hour. The measles virus is antigenically uniform. It

shares antigens with the viruses of canine distemper and bovine rinderpest.

Epidemiology

Measles is endemic throughout the world and produces epidemics every 2–3 years. The disease has maximum incidence in children between 1 and 5 years of age. It is uncommon in the first six months of life due to the presence of maternal antibodies. A **single attack confers lifelong immunity.**

- Humans are the only natural hosts of the measles virus.
- Infectivity is maximum at the prodrome (3 days prior to symptoms) and diminishes rapidly with onset of the rash.
- Spread is by direct contact with respiratory secretions and aerosols created by coughing and sneezing.
- The virus enters the body through the respiratory tract and conjunctiva. In the non-immune, infection almost always results in clinical disease.

On remote islands, the population may be highly susceptible to measles. When the virus is introduced into such communities, it may induce epidemics with high mortality.

A classical example of the above was observed in the Faroe Islands where measles appeared in 1846 after an absence of some 60 years. The epidemic spared only the very old who had been alive during the previous epidemic. When Greenland had its first exposure to the measles virus in 1951, the epidemic affected nearly all of the indigenous population.

Clinical Features

It takes about 9–11 days from the time of exposure to infection for the first signs of clinical disease to appear.

Prodromal phase This consists of malaise, fever, conjunctival injection, cough and nasal discharge. After 3–4 days of prodromal illness, a rash appears (*Clinical case*). A day or two before the rash begins, **Koplik's spots** develop on the buccal mucosa and occasionally, on the conjunctiva and intestinal mucosa. The **prodromal illness subsides within a day or two of appearance of the rash.**

Eruptive phase The red maculopapular rash of measles typically appears

on the forehead first and spreads (Fig. 49.4) downwards, to disappear in the same sequence 3–6 days later, leaving behind a brownish discolouration and finely granular desquamation.

Post-measles phase After the temperature subsides (3–4 days later) and the rash fades (in 5–6 days), the skin is discoloured (post-measles staining) and often peels off. It may continue to peel for weeks.

Fig. 49.4 Maculopapular rash on the infant's face; the hallmark of measles (*Source:* Public Health Image Library, ID 21074 / Barbara Rice / CDC)

Complications

Most patients recover uneventfully, but some may develop complications such as the following:

- **Croup, bronchitis (**due to the virus)
- **Pneumonia, otitis media** (due to secondary bacterial infection)
- **Giant cell pneumonia** (particularly in children with immunodeficiencies or severe malnutrition)
- **Meningoencephalitis**—the most serious complication; many survivors have neurological sequelae
- **Subacute sclerosing panencephalitis (SSPE)**—a rare late complication
- **Protracted diarrhea**—often seen as a complication in children in the less developed nations; the virus may be recovered from the stools of patients with measles enteritis

Complications are more common and serious in the developing

countries.

There occurs a suppression of delayed hypersensitivity after measles infection, which may last for weeks or a few months. Mantoux and other allergic skin tests may be negative during this period. **The underlying tuberculosis may become worse following an attack of measles.** Recovery from measles may also be associated with an improvement of allergic eczema or asthma, Hodgkin's disease or lipoid nephrosis.

The virus may cross the placenta and infect the fetus in maternal measles but **there is no evidence of teratogeny**. Thrombocytopenia may develop, leading to purpura and bleeding from the mouth, intestines and genitourinary tract.

Pathogenicity

The virus enters the body through the respiratory tract or the conjunctiva and multiplies locally and in the adjoining lymph nodes. The virus spreads to the reticuloendothelial system through blood. After multiplication there, a secondary viremia transports the virus to the epithelial surfaces including the skin, mouth, respiratory tract and conjunctiva.

The pathognomonic **Koplik's spots** contain giant cells, cytoplasmic and intranuclear inclusions and virion components, indicating local viral replication. Evidence of viral replication can also be seen in the vascular endothelial cells at the sites of the skin rash. **The rash represents an immune reaction between T lymphocytes and cells in which viral replication is taking place**.

During the prodromal phase, which lasts for 2–4 days, the virus can be isolated from blood, washed leucocytes, tears and respiratory secretions. It can be recovered from urine up to four days after the appearance of the skin rash.

Epidemiology

Measles is a highly contagious disease in children. Recent estimates from WHO indicate that in 2017, there were 110,000 measles deaths globally, mostly among children under the age of five. As per the WHO estimates, prior to the vaccine initiation, about 30 million cases and 700,000 deaths occur annually in developing countries. In these countries, measles is one of

the leading causes of childhood deaths, most of which follow complications such as pneumonia, diarrhea and malnutrition. The actual numbers of children affected by measles may be much higher–health workers often identify a childhood illness as simply 'pneumonia' or 'diarrhea' and may not realize that the illness is, in fact, a complication of measles (adapted from WHO's fact sheet on measles, published in November 2018). In India, the number of confirmed cases of measles in the year 2018 was 28,691 (as per the global measles update of WHO).

Laboratory Diagnosis

Diagnosis is self-evident. In atypical cases, and for differentiation from rubella, laboratory tests are useful.

1. **Specimen** Nasal secretions, throat, conjunctival swabs and blood can be used. CSF is collected in suspected cases of SSPE.

2. **Direct microscopy** A simple diagnostic test, which can be performed even before the rash appears, is the demonstration of **multinucleated giant cells in Giemsa-stained** smears of nasal secretions.

3. **IFA** The measles virus antigen can be detected in the cells of nasal secretions by immunofluorescence.

4. **Virus isolation** The virus can be isolated from the nose, throat, conjunctiva and blood during the prodromal phase and up to about two days after the appearance of the rash. The virus may be obtained from urine for a few more days. Primary human or monkey kidney and amnion cells are most useful. Cytopathic changes may take up to a week to develop, but earlier diagnosis of viral growth is possible by immunofluorescence.

5. **Serological diagnosis** Demonstration of measles-specific IgM in a single specimen of serum drawn between one and two weeks after the onset of the rash is confirmatory. A four-fold rise in titre is looked for using paired sera collected during the acute phase and 10–21 days after.

Specific neutralisation, hemagglutination inhibition (HAI) and complement-fixing antibodies (in CFT) develop early.

False negatives may occur if the serum is taken earlier than one week before or later than two weeks after the onset of the rash.

Demonstration of high-titre measles antibody in the CSF is diagnostic of SSPE.

6. **PCR** Reverse transcriptase PCR is a sensitive and specific method of diagnosis.

Prophylaxis

Passive protection

Normal human gammaglobulin or (i**mmunoglobulin** -**Ig**) given within six days of exposure as post-exposure prophylaxis can prevent or modify the disease, depending on the dose. This is useful in people who are at risk of severe illness and complications from **measles** such as infants younger than 12 months of age, pregnant women without evidence of **measles** immunity, and people with severely compromised immune systems.

Active immunisation

- The original live vaccines used the **Edmonston strain** developed by multiple passages through human kidney, amnion and chick embryo cultures.
- The **Schwartz** and **Moraten strains** are safe but effective only in children older than 15 months.
- **Edmonston–Zagreb strain**, attenuated by passage in human diploid cells, is preferred because it is able to produce seroconversion even in infants who are 4–6 months old. The recommended age for measles vaccination in developing countries is now nine months, while in the advanced nations, it remains 15 months.

The vaccine is given either by itself or in combination, like the MMR vaccine. In India, two doses of the vaccine are given, the first at 9 months and the second at around 16–24 months. **Contraindications** are immunodeficiency, untreated tuberculosis and pregnancy.

A **live attenuated vaccine** has been developed which can be given by intranasal aerosol to young babies; this gives good protection, irrespective of the presence of maternal antibodies. Efforts are underway to eradicate measles by vaccination. Considerable progress has been achieved in the USA and some other countries.

Measles eradication
India is hoping to accomplish the goal of measles elimination by the year 2020, along with other member states of the South-East Asia Region of the WHO. This will be achieved by providing high vaccination coverage with two doses of measles- and rubella-containing vaccines, by monitoring disease using effective surveillance, by being prepared for measles outbreak management and by creating awareness among the public about the importance of vaccination.

NIPAH AND HENDRA VIRUSES

In the 1990s a new genus, *Henipavirus*, was identified in an outbreak in Australia and Malaysia. Two zoonotic paramyxoviruses were found to be associated with zoonotic outbreaks. Fruit bats are their natural hosts.

Nipah virus was first identified in Kampung Sungai Nipah, Malaysia, in 1998. There was an outbreak of the disease with acute febrile illness. Pigs were found to be the intermediate hosts. It was supposed that pigs contracted the infection by eating fruits contaminated with the urine of infected fruit bats. However, in subsequent NiV outbreaks, there were no intermediate hosts. In Bangladesh in 2004, humans became infected with NiV as a result of consuming date palm sap that had been contaminated by the urine/saliva of infected fruit bats. The infection travelled to the north-eastern part of India, to the Siliguri district of West Bengal. In the Indian setting, transmission is mainly human-to-human and mainly among household contacts. The most recent outbreak, which claimed 17 lives, was reported from Kerala; transmission occurred in the hospital and close household contacts in Kozhikode and Malappuram districts. Neither the reservoir nor the vector has been detected with certainty; the involvement of fruit bats is suspected. Quick detection and containment strategies by health care authorities prevented the infection from becoming an epidemic.

Hendra virus caused the death of many horses in Australia. Some human cases were also reported. As the mortality is high with these infections, they have been classified as **Biosafety level 4 agents**.

HUMAN METAPNEUMOVIRUS

This is a respiratory pathogen, first described in 2001 by using molecular methods to identify unknown causative agents in children suffering from respiratory illness, which otherwise resembled RSV infection. Besides children, this virus can cause disease in adults with lymphomas or

leukemias and also in the elderly.

RUBELLA VIRUS

Rubella or German measles is a mild exanthematous fever characterised by transient macular rash and lymphadenopathy. In itself, the disease is trivial but rubella in pregnant women may lead to **congenital malformations** in the baby.

The teratogenic property of rubella virus was discovered by an Australian ophthalmologist Gregg, who in 1941, observed a sudden increase in congenital cataract in infants and related it to maternal rubella. Observations from different countries soon confirmed that maternal rubella induces congenital malformations of different kinds, the commonest being the triad of cataract, deafness and cardiac defects. Further progress had to wait until the rubella virus was isolated in tissue culture in 1962.

Properties

The rubella virus is a pleomorphic, roughly spherical particle, 50–70 nm in diameter, with a single-stranded RNA genome and surrounded by an envelope carrying hemagglutinin peplomers. Rubella virus are released by budding from the host cell surface to be freed into the host's system (Fig. 49.5). It agglutinates goose, pigeon, one-day-old chick and human erythrocytes at 4°C. Structurally and in many other features, it resembles togaviruses. The rubella virus has been classified in the family Togaviridae as the only member of the genus ***Rubivirus***.

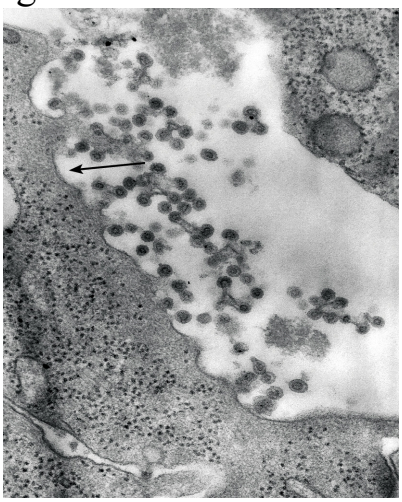


Fig. 49.5 Transmission electron microscopic (TEM) image shows the presence of rubella virus, virions, in the process of budding (arrow) from the host cell surface to be freed into the host's system (*Source:* Public Health Image Library, ID 10221 / Dr. Fred Murphy; Sylvia

Whitfield / CDC)

Inactivation of rubella
The virus is inactivated by ether, chloroform, formaldehyde, beta-propiolactone and desoxycholate. It is destroyed by heating at 56°C but survives for several years at –60°C.

The experimental model for the teratogenic effects of rubella is the pregnant rabbit, in which the virus infects the fetus transplacentally, leading to congenital malformations.

Clinical Features

Infection is acquired by inhalation.

After an **incubation period** of 2–3 weeks, a generalised rash develops on the face, spreading to the neck, trunk and extremities, sparing the palms and soles. The rash is generally discrete and disappears by the third day. There is non-tender enlargement of the posterior cervical glands. Koplik spots, seen in measles, are absent. The disease occurs principally in children but may affect all ages.

Complications

The common complications are arthralgia and arthritis, commoner in women and with increasing age. Based on the results of in vitro studies, rubella infection is presumed to cause chromosomal breakages and inhibition of mitoses in infected embryonic cells.

The virus can be recovered from the throat up to seven days before the rash appears. Viremia has been demonstrated as early as the seventh day before the rash and ceases shortly after the appearance of the rash. The virus can also be demonstrated in feces and urine. Some patients develop subclinical infection and are infectious to others. Infection of the fetus is through the maternal bloodstream.

Congenital rubella

Fetal damage caused by maternal rubella is related to the stage of pregnancy.

- *Very early pregnancy* Infection at this stage ends in abortion.

- *First trimester* There is a reported risk of up to 90 per cent of occurrence of congenital malformations.
- *Later on in pregnancy* The damage caused may be more subtle, in the form of communication defects or developmental retardation and may not be apparent until the child grows older.

Congenital rubella syndrome

The commonest malformations caused by rubella are cardiac defects, cataract (Fig. 49.6) and deafness. Several other features have been recognised in babies with congenital rubella, including hepatosplenomegaly, thrombocytopenic purpura, myocarditis and bone lesions, constituting the **'expanded rubella syndrome'**.

The rubella virus is present in all excretions of congenitally infected infants. About a third of them continue to shed the virus for six months, and a few for a year or more. The virus may persist in tissues such as cataractous lenses for several years. Infected babies constitute an important source of infection to the staff in nurseries.

Fig. 49.6 Congenital rubella in a child—**bilateral cataract** (*Source:* Public Health Image Library, ID 4284 / CDC)

Laboratory Diagnosis

Routine diagnosis of rubella is not required, but laboratory confirmation becomes important when rubella is suspected in pregnant women.

Diagnosis in pregnancy

Diagnosis can be established by serology or virus isolation.

Serology Serological diagnosis is the mainstay of diagnosis. ELISA to detect IgM and IgG antibodies gives valuable information. A finding of IgM antibodies alone, without IgG, means current acute infection. IgG antibody

alone without IgM means past infection or vaccination and denotes immunity. Paired serum sample is tested 10 days apart to demonstrate a significant rise or fall in antibody titre. A screening test for pregnant women by TORCH panel (Toxoplasmosis, Rubella, Cytomegalovirus and Herpes) is done to rule out the possibility of congenital infections.

Cultivation The virus can be grown in many primary cell cultures and continuous cell lines, such as rabbit kidney (RK 13), baby hamster kidney (BHK 21) and Vero. Cytopathic changes develop only in a few cell lines such as RK 13. In others, virus growth can be identified by interference, using a challenge virus such as ECHO 11. Rubella virus replicating in the cells are released by budding from the cell surface.

Rubella virus isolation is not commonly used for diagnosis because of the difficulties and delay involved. The virus may be isolated from blood during the early stage or more successfully, from throat swabs in rabbit kidney or Vero cells. The virus grows better if cultures are incubated at a lower temperature, such as 33–35°C.

Diagnosis of congenital rubella

Serology IgM antibodies in the newborn indicate intrauterine infection. IgM does not cross the placenta and thus, indicates the response of the fetus to the infection. If diagnosis is needed at a later time, paired serum from the mother and child can be used to demonstrate IgG antibodies. In congenital infection, the IgG antibodies will be present in the newborn beyond six months. If they are passively transferred from the mother, levels will decline in the newborn while remaining the same in the mother.

Isolation In congenital rubella, the virus may be isolated from a variety of sources such as urine, throat swabs, leucocytes, bone marrow or cerebrospinal fluid.

Prophylaxis

Rubella infection confers lasting immunity as the virus has only one antigenic type. Re-infections have, however, been reported.

Live attenuated vaccines have been developed by serial passage of the virus in tissue culture. The vaccine in use today is the RA 27/3 strain grown in human diploid cell culture and administered by subcutaneous injection, alone or in combination with measles and mumps components as the **MMR**

vaccine. The vaccine is generally well tolerated, though minor reactions like lymphadenopathy, rash and arthralgia may sometimes occur. It should not be given to immunodeficient subjects. Pregnancy is an absolute contraindication and should be avoided for three months after vaccination. The vaccine virus is apparently not teratogenic. Inadvertent administration of the vaccine to pregnant women may not, therefore, lead to congenital defects in the baby.

Epidemiology

Rubella is worldwide in distribution. Serological surveys in different countries have shown that 80–90 per cent are immune by the age of 15 years. About 10–20 per cent of mothers are non-immune and, therefore, vulnerable.

Key points

- Paramyxoviridae is a family of enveloped, helical, RNA viruses which are 100–300 nm in diameter. There are four genera: *Rubulavirus*, *Paramyxovirus*, *Morbillivirus* and *Pneumovirus*.
- Paramyxovirus is characterised by the presence of both hemagglutinin and neuraminidase.
- Parainfluenza viruses types 1–4 cause upper respiratory infections.
- Newcastle disease virus is of great economic importance.
- The mumps virus belongs to *Rubulavirus* and causes mumps and aseptic meningitis. This virus may also be the most common cause of aseptic meningitis. Vaccination in childhood with a live attenuated virus (Jeryl–Lynn strain) gives long-lasting immunity.
- The respiratory syncytial virus (RSV) differs from other members of the family in lacking both hemagglutinin and neuraminidase. It commonly infects children, causing a common cold-like illness. Infection during the first year of life may induce a severe, life-threatening bronchiolitis. Two types of RSV, A and B, can be detected.
- Antibodies to RSV can be demonstrated in the serum; the presence of rising antibody titres or high IgM titres in a single serum sample is diagnostic. Aerosolised ribavirin can be used to treat RSV infections, if indicated.
- Morbillivirus includes the measles virus, which causes measles. Diagnosis is by virus isolation in cell culture, by serological methods which demonstrate a rise in serum antibody titres or PCR. Vaccination in childhood with a live attenuated virus gives immunity that should be boosted at 4–6 years of age.
- A new genus, *Henipavirus,* has been recently identified with the Nipah and Hendra viruses causing zoonotic outbreaks.

- Human metapneumovirus causes respiratory illnesses in children and diseases in adults with lymphoma and leukemia.
- Rubella (German measles) is a mild erythematous fever, characterised by transient macular rash and lymphadenopathy (sometimes arthralgia). Rubella in pregnant women may cause congenital malformations in the baby.
- Rubella virus (family Togaviridae) has a single-stranded RNA genome and an envelope with hemagglutinin peplomers.
- Infection is acquired by inhalation. The incubation period is 2–3 weeks.
- The virus may spread to the fetus through the bloodstream, causing death or congenital malformations.
- In classical congenital rubella syndrome, cardiac defects, cataract and deafness are seen; hepatosplenomegaly, thrombocytopenic purpura, myocarditis and bone lesions can occur.

Questions

Essay

1. Explain the pathogenesis and complications of mumps virus infections.

Short Answers

1. RSV
2. Immunisation against mumps and measles
3. Nipah and Hendra viruses
4. Metapneumovirus
5. SSPE
6. Congenital rubella

CHAPTER 50

Arthropod- and Rodent-Borne Viral Infections

Chapter navigation

- Togaviridae
- Flaviviridae
  - Mosquito-borne group
  - Tick-borne group
- Bunyaviridae
- Reoviridae
- Ungrouped arboviruses
- Rodent-borne viruses

ARTHROPOD-BORNE VIRUSES

Arboviruses (**arthropod-borne viruses**) are a **varied group of RNA** viruses that have **common ecological and epidemiological attributes** but diverse physical and chemical properties. They multiply in, and are **transmitted by hematophagous insect vectors** to vertebrates, including humans. Insect viruses and viruses of vertebrates that are sometimes mechanically transmitted by insects do not come under this category.

Arboviruses are classified according to their physical and chemical features into taxonomical families named Togaviridae, Flaviviridae, Bunyaviridae, Reoviridae, Arenaviridae and Filoviridae (Table 50.1). Within each family, they are classified into genera and antigenic groups based on serological relationships. Some viruses are ungrouped.

Table 50.1 Taxonomy of some important arboviruses

Family	Genus	Important species
Togaviridae	*Alphavirus*	Chikungunya, O'nyong-nyong, Mayaro, Semliki forest, Sindbis, Ross River, Eastern, Western

		and Venezuelan equine encephalitis viruses
Flaviviridae	*Flavivirus*	Japanese encephalitis, Murray Valley encephalitis, West Nile, Ilheus, St. Louis encephalitis, yellow fever, dengue types 1, 2, 3, 4, Russian spring-summer encephalitis complex, louping ill, Powassan, Zika virus, Kyasanur Forest disease, Omsk hemorrhagic fever
Bunyaviridae	*Bunyavirus*	California encephalitis, Oropouche, Turlock
	Phlebovirus	Sandfly fever viruses, Rift Valley fever virus
	Nairovirus	Crimean Congo hemorrhagic fever viruses, Nairobi sheep disease virus, Ganjam virus
	Hantavirus	Hantan, Seoul, Puumala, Prospect Hill, Sin Nombre viruses
Reoviridae	*Orbivirus*	Colorado tick fever, African horse sickness, Bluetongue viruses
Arenaviridae	*Arenavirus*	Junin, Machupo, lymphocytic choriomeningitis and Lassa fever
Filoviridae	*Marburgvirus*	African hemorrhagic fevers, Ebola virus

Arboviruses are worldwide in distribution but are far more numerous in the tropical than in the temperate zones. Over 500 viruses have been listed; most cause silent infections in rodents and other wild mammals but about 100 of them can infect humans. In India, over 40 arboviruses have been detected, of which more than 10 are known to produce human disease. Natural cycles of the virus involve infected non-human vertebrate hosts including many species of animals and birds. The virus is transmitted by arthropods.

Arthropod vectors The ability to multiply in arthropods is a special characteristic of arboviruses. The most common arbovirus vectors are:

- Mosquitoes
- Ticks

The less common vectors are:

- *Phlebotomus*
- *Culicoides*

- *Cimicidae*

Antigenic Structure

Three antigens are important in serological studies: **hemagglutinins, complement-fixing and neutralising antigens**—all integral parts of the virus particle. Considerable antigenic cross-reactions occur among arboviruses. The **plaque reduction neutralisation test (PRNT)** shows the greatest specificity for the identification of the viruses.

Hemagglutination Most arboviruses agglutinate the red cells of day-old chicks or geese. Hemagglutination is influenced by pH and temperature, the optimal requirements varying with different viruses. Spontaneous elution does not occur. Hemagglutination is inhibited specifically by antibodies; this can be used in diagnostic assays and non-specifically by lipoprotein inhibitors in serum, brain and other tissues.

Resistance

In general, arboviruses are labile, being readily inactivated at room temperature and by bile salts, ether and other lipid solvents. Infectivity may be retained at –70°C or by lyophilisation.

Pathogenesis

The life cycle of the arbovirus is based on its ability to replicate in the arthropod vector and the primate host. The infected, viremic, bloodsucking vector is responsible for the transmission of the virus. In the vector, the virus multiplies in the gut and spreads to other organs including the salivary glands. High viremia allows the virus to be transmitted in very low volumes of the saliva of the bloodsucking female vector. Several arthropods are responsible for the transmission of infections
(Table 50.2).

Table 50.2 Reservoir hosts, geographic distribution and vectors of arboviruses and rodent-borne viruses associated with different clinical syndromes

Virus	Genus	Reser-voir/ hosts	Geographic distribution	Vector
Encephalitis				

Virus	Genus	Reser-voir/ hosts	Geographic distribution	Vector
Eastern equine encephalitis	*Alphavirus*	Birds	Americas	Mosquito
Western equine encephalitis	*Alphavirus*	Reptiles	Americas	Mosquito
Venezuelan equine encephalitis	*Alphavirus*	Rodents	Americas	Mosquito
St. Louis encephalitis	*Flavivirus*	Birds	Americas	Mosquito
West Nile	*Flavivirus*	Birds	Africa, Europe, USA, West Asia	Mosquito
Japanese encephalitis	*Flavivirus*	Birds	East and Southeast Asia	Mosquito
Murray Valley encephalitis	*Flavivirus*	Birds	Australia	Mosquito
RSSE complex	*Flavivirus*	Rodents, other mammals, birds, ticks	East Europe, the (former) USSR	Tick
Louping ill	*Flavivirus*	Sheep	Britain	Tick
Powassan	*Flavivirus*	Rodents	North America	Tick
California	*Bunyavirus*	Rodents	North America	Mosquito
Hemorrhagic fever				
Chikungunya	*Alphavirus*	Not known	Africa, Asia	Mosquito
Dengue, types 1–4	*Flavivirus*	Not known	Widespread, especially in Asia and the Caribbean the tropics	Mosquito
Yellow fever	*Flavivirus*	Monkeys, humans	Africa, South America	Mosquito
Kyasanur Forest disease	*Flavivirus*	Rodents, shrews, monkeys and ticks	India	Tick
Omsk hemorrhagic fever	*Flavivirus*	Small mammals	The (former) USSR	Tick
Crimean Congo	*Nairovirus*	Small mammals	The (former) USSR,	Tick

Virus	Genus	Reser-voir/ hosts	Geographic distribution	Vector
			Central Asia	
Febrile Illness with or without rash (non-hemorrhagic) and arthralgia				
Ebola virus	*Filoviridae*	Chimpanzees, gorillas, fruit bats, monkeys, forest antelope and porcupines	Mainly localised to African countries like Sudan, Zaire, Uganda and Congo where it continues in several pockets Outbreaks have occurred in other Western countries: Italy, Spain, UK & US	Not an arbovirus in the true sense as there are no arthropods incriminated in its transmission
O' nyong-nyong	*Alphavirus*	Not known	Africa	Mosquito
Ross River	*Alphavirus*	Small animals	Australia	Mosquito
Sindbis	*Alphavirus*	Birds, mammals	Africa, Asia	Mosquito
Mayaro	*Alphavirus*	Monkeys, marsupials	South America	Mosquito
West Nile	*Flavivirus*	Birds	Asia, Africa, USA	Mosquito
Sandfly fever	*Bunyavirus*	Not known	The Mediterranean, Asia, tropical parts of America	Sandfly
Rift Valley fever	*Bunyavirus*	Sheep, cattle	Africa	Mosquito
Oropouche	*Bunyavirus*	Not known	South America	Mosquito
Colorado tick fever	*Orbivirus*	Rodents	USA	Tick
Rodent-borne hemorrhagic fevers				
Hantavirus pulmonary syndrome	*Hantavirus*	Deer mouse	South America and the United States	Deer mice
Hemorrhagic fever with renal syndrome (HFRS)	*Hantavirus*	Rodents	China, Russia and Korea	Rodents

Virus	Genus	Reser-voir/ hosts	Geographic distribution	Vector
Lassa fever	*Lassavirus*	Rodents	Western Africa	Rodents

Humans are generally dead-end hosts as the concentration of the virus in the blood is low and the viremic period is short. In some infections like dengue and yellow fever, the viremia is high, hence humans act as reservoirs of the virus and can transmit.

In humans, the infection may be associated with more than one syndrome after the virus enters the body through the bite of the insect vector. After multiplication in the reticuloendothelial system, viremia of varying duration ensues and, in some cases, the virus is transported to the target organs such as the central nervous system in encephalitis, the liver in yellow fever and the capillary endothelium in hemorrhagic fevers.

Three main clinical entities of arboviruses

1. Fevers with or without rashes
2. Hemorrhagic fevers
3. Encephalitis

Case fatality in hemorrhagic fevers and encephalitis is often high. All infections occur with varying degrees of severity, subclinical infections being common. Arboviruses also cause a number of veterinary diseases such as Eastern, Western and Venezuelan equine encephalitis in horses in America, Rift Valley fever in sheep and cattle in Africa, bluetongue in donkeys in India, Africa and America, Ganjam disease of sheep in India and African horse sickness in horses and mules in Africa and Asia.

Epidemiology

Arbovirus infections are zoonoses (with a few possible exceptions such as dengue and O'nyong-nyong) maintained in nature by animals in a vector cycle, with human infection occurring when individuals come into contact with the vector either accidentally or when they live in close proximity with the animal-vector cycle. In some areas, the arbovirus infection appears to be seasonal, and outbreaks are known to occur periodically. The reason for this

could be an increase in the vector population or reintroduction of the virus to the area through migratory birds or other reservoirs.

The epidemiology of arbovirus infections has **two patterns**:

i) It is linked with the ecology of the arthropod vectors and vertebrate hosts. Most arboviruses exist in nature in animal or avian species, in which infection is asymptomatic. The vector arthropod gets infected by biting a viremic vertebrate. The vector becomes infective only after the incubation period (extrinsic incubation period). Human disease results when the virus is accidentally transmitted to humans, either directly by the vector or through the intermediary of animal reservoirs.

ii) A second epidemiological pattern is seen in diseases like dengue where no non-human vertebrate host has been identified. Here, the virus is maintained in a cycle composed of humans and the domestic mosquito. Transovarial transmission in ticks maintains the virus in the vector.

Control

Control measures are indicated only in those infections that lead to epidemics or epizootics. These consist essentially of vector control.

A vaccine against yellow fever has been successful and is routinely used for travellers to Africa and other endemic areas. Vaccines against Japanese encephalitis (JE) have also been used during outbreaks or in endemic areas. However, it has not been used in the control of other arbovirus diseases. Vaccines have been used against Japanese encephalitis in Asia and the US. A live attenuated SA-14-14-2 vaccine against JE is used in some endemic areas in Asia including India. Due to large epidemics, the WHO has initiated vaccine trials against dengue. Trials with tetravalent dengue vaccine are ongoing in India.

Laboratory Diagnosis

Diagnosis may be established by virus isolation or serology. Strict biosafety measures are required to carry out virus isolation.

1. **Specimen** As all arbovirus infections are viremic, blood collected during the early phase of the disease may yield the virus. Isolation may also

be made from the cerebrospinal fluid (CSF), in some encephalitic cases, and tissues.

2. **Virus isolation** In the laboratory, mice are commonly used for growing arboviruses, intracerebral inoculation in suckling mice being the most sensitive method for their isolation. Specimens are inoculated intracerebrally into suckling mice. The animals develop fatal encephalitis, though serial blind passages may be necessary in some cases. Arboviruses can be grown in the yolk sac or chorioallantoic membrane of chick embryo, in tissue cultures of primary cells like chick embryo fibroblasts or continuous cell lines like Vero or HeLa, and in cultures of appropriate insect tissues.

Some viruses, mainly alpha and flavivirus, may also be isolated in cell cultures namely, HeLa, BHK, MRC-5 and Vero. **Mosquito cell lines have been used for isolation.** Isolates are identified by hemagglutination inhibition, complement fixation, immunofluorescence, immunochromatography, ELISA or neutralisation with appropriate antisera. Isolation of the virus from insect vectors, birds or animals in reservoir areas indicates arbovirus activity in the area.

3. **Serology** Diagnosis may also be made by demonstrating IgM antibodies in acute phase by ELISA and immunochromatographic methods, or rise in IgG titres in paired serum samples (first sample taken soon after onset and a second sample 2–3 weeks later) to demonstrate a four-fold rise in titre, suggestive of ongoing or recent infection.

Serological diagnosis is often complicated due to the antigenic cross-reaction between related viruses (e.g., cross-reaction between *Alphavirus* and *Flavivirus* groups).

Detection of viral proteins and antigens, or RNA by molecular methods (conventional or real-time PCR) are more specific and rapid. They have replaced conventional CFT, HAI and neutralisation tests.

TOGAVIRIDAE

Morphology

Togaviruses are spherical, enveloped viruses with a diameter of 50–70 nm. The genome is a molecule of single-stranded RNA. The virus replicates in

the host cell cytoplasm and is released by budding through host cell membranes. The name *Togavirus* is derived from *toga*, meaning the Roman mantle or cloak, and refers to the viral envelope.

Classification

The family Togaviridae contains arboviruses belonging to the genus *Alphavirus*. Rubella virus of the genus *Rubivirus* is also a togavirus but is not arthropod-borne and differs in its epidemiology and other features (discussed in Chapter 49).

Alphavirus

The genus *Alphavirus* contains 32 species, of which, at least 13 are known to infect humans. **All of them are mosquito-borne.** They exhibit cross-reaction in HAI and CFT but not in neutralisation tests.

They produce epidemics of encephalitis in America and dengue-like fever in the tropics.

i. Encephalitis Viruses Group

Three members of this group—Eastern, Western and Venezuelan equine encephalitis viruses—cause encephalitis in horses and humans. **Eastern equine encephalitis (EEE)** occurs along eastern Canada, USA and the Caribbean, causing sporadic cases and small epidemics. **Western equine encephalitis (WEE)** is more widely distributed in America and causes large epidemics. **Venezuelan equine encephalitis (VEE**), prevalent in Central and South America, usually causes an influenza-like illness, with encephalitis in a small proportion of cases. Several species of *Culex* and *Anopheles* mosquitoes are the vectors, and wild birds the reservoirs. Formalinised vaccines have been developed for EEE and WEE and a live attenuated vaccine for VEE is available for horses but not humans.

ii. Fever with Arthritis Group

Chikungunya virus (CHIKV)

This virus was first isolated from human patients in Tanzania. The vectors *Aedes aegypti* and *A. albopictus* are found in endemic areas. A mutation of CHIKV in the E1 protein enabled this virus to eliminate its cholesterol

dependence. This resulted in better viral uptake, replication and transmission by *A. albopictus*. This could have caused the outbreak of CHIKV infection in India in 2004–2006. *A. albopictus* has a wider geographical distribution than *A. aegypti*.

No animal reservoir has been identified. The name 'chikungunya' is derived from the native Makonde word for the disease in which the patient lies 'doubled up' due to severe joint pain. Epidemics of chikungunya have occurred in many African countries. **The virus first appeared in India in 1963**, when along with dengue, it caused extensive epidemics in Kolkata, Chennai and other areas. Chikungunya **outbreaks occurred at irregular intervals** along the east coast of India and in Maharashtra till 1973. Since then, the virus had been quiescent until it reappeared in 2004–2006. There was a large outbreak that began in Andhra Pradesh and Tamil Nadu, which spread to Kerala, Karnataka and Delhi, affecting nearly a million people.

Genotypes There are three major chikungunya virus genotypes:

- West African
- East/Central/South African (ECSA)
- Asian genotypes

They are generally restricted to these geographic areas. However, spread of ECSA to Asian regions has been reported.

Reasons for re-emergence The disease appears in epidemics after a gap of a decade or two. This is attributed to (i) mutation of the virus and (ii) wider distribution of the vector *Aedes albopictus*. During interepidemic periods, the virus is thought to be maintained in the sylvatic cycle in non-human primates.

Symptoms The disease presents with a sudden onset of fever and crippling joint pain, especially in the ankles and wrists. Joint involvement is bilateral and symmetric. Migratory polyarthritis is also seen in some individuals. Lymphadenopathy and conjunctivitis may also be present. A few patients have leucopenia with elevated aspartate aminotransferase (AST) and a positive C-reactive protein may be seen in some patients. A maculopapular rash is common. Appearance of the rash often coincides with defervescence. Hemorrhagic manifestations are rare. The fever is typically

biphasic with a period of remission of 1–6 days. Recovery takes weeks while some older patients experience joint pain and stiffness for several years. Persistence may be seen in HLA B27 individuals. Clinically, chikungunya cannot be differentiated from uncomplicated dengue. Laboratory tests based on IgM detection can help in confirming chikungunya.

Diagnosis A patient is suspected to have CHIKV only if she meets the clinical criteria. A confirmed (definitive) case is defined as a patient who meets both the clinical and laboratory criteria.

Clinical criteria for diagnosing CHIKV

The clinical criteria for diagnosing CHIKV infection include acute onset of fever and severe arthralgia/arthritis with or without skin rash and a history of residence in an epidemic area or having been in and subsequently having left an epidemic area 15 days before the onset of symptoms.

Laboratory criteria The following tests detect the virus or antibodies to confirm CHIKV.

- Virus isolation/presence of viral RNA is detected by reverse transcriptase PCR.
- Presence of virus-specific IgM antibodies in a single serum sample collected in the acute or convalescent stage or four-fold increase in IgG values in samples collected at least 3 weeks apart by ELISA or other immune assays confirms CHIKV.

Treatment Supportive management and physiotherapy for relief of joint pains are the only options. No vaccine is available to prevent the infection.

O'nyong-nyong virus, Semliki Forest virus and Sindbis virus

They are the other alphaviridae that are mosquito-borne and produce febrile illness in humans. They are localised to Uganda, Africa. Sindbis virus has been recovered from the Philippines and Australia and antibodies have been detected in some human sera in India.

FLAVIVIRIDAE

The family Flaviviridae contains only one genus, *Flavivirus*. They are smaller than alphaviruses, being 40 nm in diameter. The name *Flavivirus* refers to the yellow fever virus (*flavus* in Latin means yellow).

There are over 60 arthropod-borne flaviviruses. Representative members of this group are distributed in all parts of the world, covering all the

zoogeographic regions. They may be considered under two groups: **mosquito-borne** and **tick-borne viruses.**

Mosquito-Borne Group

Encephalitis viruses

The following five members of this mosquito-borne group cause encephalitis, each of them limited to a geographic zone.

1. ***St. Louis encephalitis virus*** This is prevalent in North and Central America and is the most important mosquito-borne disease in the USA. Wild birds act as the reservoir and *Culex tarsalis* as the vector.
2. ***Ilheus virus*** This occurs in South and Central America, and is maintained in forests by a cycle of mosquitoes, wild birds and monkeys. Human infection is largely subclinical. Encephalitis is rare.
3. ***West Nile virus*** This virus was originally isolated in 1937 from the West Nile province of Uganda. It has since been reported from African countries, Israel, Cyprus, France and India. It causes a dengue-like illness in humans and encephalitis in horses. The virus is maintained in nature in wild birds. In India, the virus has been isolated from *Culex* mosquitoes and from febrile as well as encephalitic patients from Rajasthan to Karnataka. In 1999, West Nile fever appeared in New York, and since then, the virus has spread across much of the USA, becoming a major public health problem.
4. ***Murray Valley encephalitis virus*** This is confined to Australia and New Guinea. The virus is believed to occur normally in an enzootic cycle involving wild birds and mosquitoes, and to break out only occasionally into epidemics. *Culex annulirostris* is the vector.
5. ***Japanese encephalitis (JE)***

   Geographical distribution This virus is quite widespread—from East Asia to South Asia; from Korea and Japan in the north and to India and Malaysia in the south. The disease was first recognised in Japan in 1935 and was named Japanese 'B' encephalitis to distinguish it from 'encephalitis A' (encephalitis lethargica, von Economo's disease). Several large-scale epidemics have occurred since then. Epidemics show seasonal incidence (summer–autumn) in the temperate regions, though this is not evident in the tropical areas. *Culex tritaeniorhynchus*, a rural mosquito

that breeds in rice fields, is the principal vector.

Clinical features Japanese encephalitis virus causes the most serious clinical disease in this group. The disease has an abrupt onset with fever, headache and vomiting. After 1–6 days, signs of encephalitis set in with nuchal rigidity, convulsions, altered sensorium and coma. The fever is high and continuous. There is presence of neutrophil leucocytosis in peripheral blood and pleocytosis with normal or raised sugar levels and slightly raised protein levels in the CSF. The mortality rate in some epidemics has been up to 50 per cent. Convalescence may take several weeks. Up to 50 per cent of survivors may have neurological or psychiatric sequelae. Infection in pregnant women during the first and second trimester has led to fetal death. The large majority of infections are, however, asymptomatic and it has been estimated that 500–1,000 inapparent infections occur for every case of clinical disease.

JE in the Indian setting In India, Japanese encephalitis was first recognised in 1955 in Pondicherry by Lappeyssonnie L., a French doctor. The virus was isolated from mosquitoes of the *Culex vishnui* complex from Vellore during an outbreak of encephalitis in Tamil Nadu. The virus continues to be active in Tamil Nadu and Andhra, causing illness mainly in children, indicating the endemic nature of the virus. Most of the cases occur between October and November.

Since 1976, the highest rates of human disease have been reported from the states of Andhra Pradesh, Assam, Bihar, Haryana, Goa, Karnataka, Kerala, Tamil Nadu, Pondicherry, Uttar Pradesh and West Bengal. In addition, sporadic cases have been reported from different parts of the country, excepting Dadra, Daman, Diu, Gujarat, Himachal Pradesh, Jammu and Kashmir, Lakshadweep, Meghalaya, Nagar Haveli, Punjab, Rajasthan and Sikkim. Japanese encephalitis has become a major public health problem of national importance in India.

Epidemiology **Herons act as reservoir hosts** and **pigs as amplifier hosts** (Fig. 50.1). Human infection is a 'dead-end' event and occurs when the infected mosquitoes reach high density. The natural cycle in India also may be similar. Cases are reported throughout the year in India, more so during the rainy season. Natural infection has been demonstrated in *Ardeid* birds (herons and egrets); bird-to-bird transmission also takes place

through *Culex tritaeniorhynchus*. Other birds such as ducks, pigeons and sparrows may also be involved. Vertebrate hosts may include cattle and buffaloes (besides pigs).

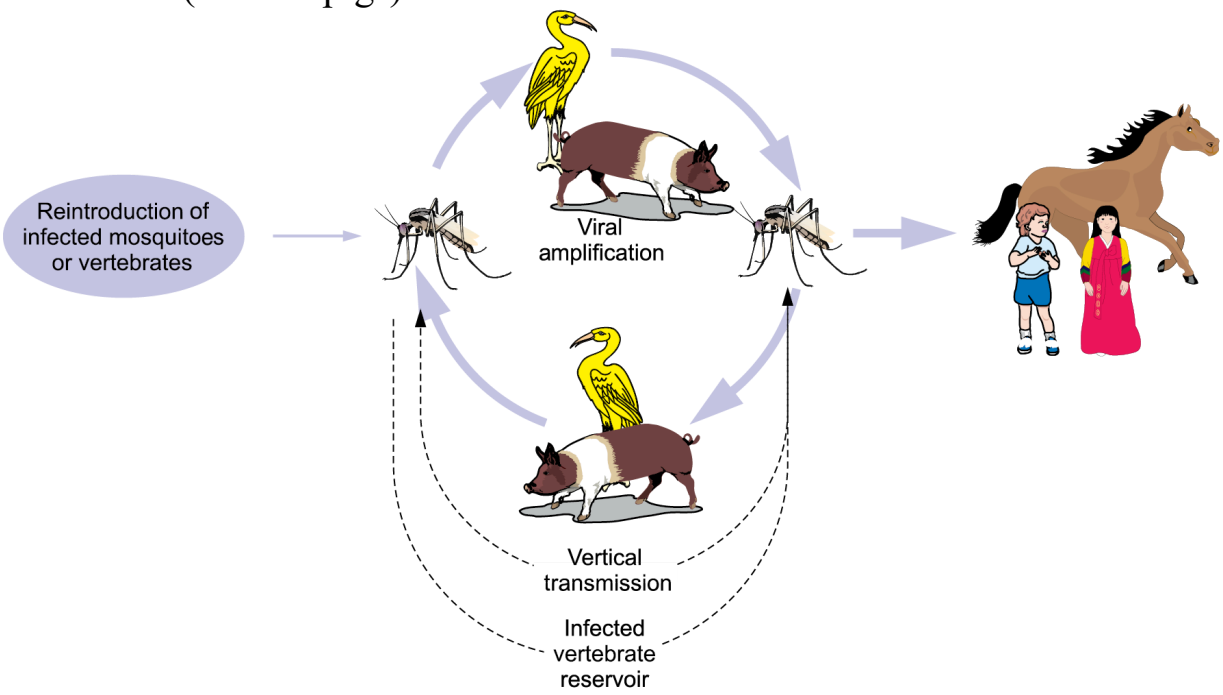


Fig. 50.1 Transmission cycle of Japanese encephalitis virus

The mosquito *Culex tritaeniorhynchus* has a predilection for cattle and bites them in preference to humans or pigs but cattle do not develop viremia, hence do not spread the virus. Pigs are the amplifier hosts. The high cattle–pig ratio in India has been suggested to be a factor limiting human infection.

Control measures Preventive measures include mosquito control and locating piggeries away from human dwellings.

Vaccine

- A **formalin-inactivated mouse brain vaccine** using the **Nakayama strain** has been used successfully for immunisation in Japan and, on a small scale, in India as well. Two doses administered two weeks apart, followed by a booster 6–12 months later, constitute a full course. The immunity produced by the vaccine is short-lived.
- A **live attenuated vaccine** has been developed in China from the **JE strain SA 14-14-2**, passed through weanling mice. It is

administered in two doses, one year apart. The vaccine has reportedly been effective in preventing clinical disease.

Vaccines licensed in India
- Inactivated Vero cell culture-derived SA-14-14-2
- Inactivated Vero cell culture-derived Kolar strain, 821564XY, JE vaccine

These are recommended for children living in endemic areas.

The vaccination of pigs has been proposed in view of their importance as amplifier hosts.

Yellow fever

Yellow fever was recognised as a clinical entity as early as the 17th century but since the early 20th century, the disease has largely been confined to certain areas of Africa and South and Central America.

Clinical features After an incubation period of 3–6 days, the disease starts as a fever of acute onset with chills, headache, nausea and vomiting. The pulse is usually slow despite a high temperature. Jaundice, albuminuria and hemorrhagic manifestations develop and the patient may die of hepatic or renal failure. Most cases are less severe, especially in the endemic areas, and may present as undifferentiated fever without jaundice.

Epidemiology The disease occurs in two distinct patterns:

1. In the **urban cycle,** humans act as the natural reservoir, the virus being transmitted by the domestic *Aedes aegypti* mosquito.
2. In the forest or **sylvatic cycle**, wild monkeys act as the reservoirs and forest mosquitoes (*Haemagogus spegazzinii* in South America and *Aedes africanus* and *A. simpsoni* in Africa) as the vectors. Human cases occur only when humans trespass into the forest or when the monkeys raid villages near the forest.

Control Yellow fever could be controlled by eradicating the vector mosquito but this is obviously impracticable with the concomitant existence of sylvatic disease.

A safe and effective vaccine, the non-neurotropic **17D vaccine**, is administered by subcutaneous inoculation. Vaccination is mandatory for travel to or from endemic areas. It is valid for 10 years, beginning 10 days after vaccination. In India, the 17D vaccine is manufactured at the Central Research Institute, Kasauli.

Geographical distribution Yellow fever is largely confined to Central and South America and Africa. India is a yellow fever 'receptive' area but the disease **does not exist in India.** Hence it is important to prevent the establishment of the disease. India offers a receptive area with a large population of *Aedes aegypti* and non-immune humans. Strict vigilance is enforced on vaccination and quarantine for travel from endemic areas. This has checked the entry of the virus into India through legitimate passengers. It is likely that any stray virus introduced may have been kept out by the prevalence in the local *Aedes aegypti* of the dengue virus, and of antibodies to a wide range of arboviruses in the local population. Another reason could have been that in Africa, yellow fever was mainly in the west, and in India, *Aedes* mosquitoes were common along the east coast, so that even stray importations of the virus by sea may not have found suitable vectors. This is no longer valid as yellow fever has, in recent years, caused epidemics in East Africa, and *Aedes* mosquitoes have spread all along the west coast of India. If yellow fever gets established in India, the consequences could be catastrophic due to the large vulnerable, non-immune population.

Dengue virus

Clinical Case

A 40-year-old man presented to the medical OPD with high fever, severe headache and pain around the eyes for the past five days. On examination, petechial lesions were noted on his forearm. The tourniquet test (to detect the fragility of the capillaries) and dengue NS1 antigen capture ELISA were positive. His platelet count was 30,000/mm^3. The patient recovered with supportive management. The case was notified to the District Health Department.

The dengue virus is widely distributed throughout the tropics and subtropics (in Swahili, *Ki denga pepo* means a sudden seizure by a demon). The term '**break-bone fever'** was coined during the Philadelphia epidemic in 1780. Dengue fever is clinically similar to the illness caused by the chikungunya and O'nyong-nyong viruses. Four types of dengue virus exist: DEN 1, first isolated from Hawaii in 1944, DEN 2 from New Guinea in 1944 and DEN 3 and 4 from the Philippines in 1956. Dengue has been on the rise worldwide over the last few decades and today, ranks as the most important vector-borne disease, with about 2.5 billion people in 200 countries at risk.

Clinical findings Dengue manifests after an incubation period of 3–14

days.

a) **Febrile phase** Patients typically develop a high-grade fever of sudden onset with headache, retrobulbar pain, photophobia, accompanied by facial flushing, skin erythema and pain in the back and limbs (break-bone fever), lymphadenopathy and maculopapular rash. The fever is typically biphasic (saddleback) (*Clinical case*). This acute febrile phase usually lasts 2–7 days and is often characterised by generalised body ache, myalgia, arthralgia, rubeliform exanthema and headache.

b) **Critical phase** Patients become worse around the time of defervescence, when the temperature drops to 37.5–38°C or less and remains below this level, usually on days 3–8 of illness. Progressive leukopenia followed by a rapid decrease in platelet count usually precedes plasma leakage. An increasing hematocrit above the baseline may be one of the earliest additional signs.

c) **Complications** with hemorrhagic manifestations (**dengue hemorrhagic fever**) or with shock (**dengue shock syndrome**) can occur in persons who have non-neutralising heterologous antibodies from a previous infection or in infants with maternal antibodies. These complications, first recognised in Thailand, have since occurred in many countries in Western Pacific, Southeast Asia including India. They are also common in previously healthy children in the indigenous populations of endemic areas.

Dengue virus is transmitted from person to person by *Aedes aegypti* mosquitoes. The extrinsic incubation period is 8–10 days. No vertebrate hosts other than humans have been identified.

Clinical criteria for dengue fever, dengue hemorrhagic fever and dengue shock syndrome

Dengue fever (DF) An acute febrile illness with two or more of the following manifestations: headache, retro-orbital pain, myalgia, arthralgia, rash and hemorrhagic manifestations.

Dengue hemorrhagic fever (DHF) Clinical criteria of dengue fever plus hemorrhagic tendencies evidenced by one or more of the following:

- Positive tourniquet test
- Petechiae, ecchymoses or purpura
- Bleeding from mucosa, gastrointestinal tract, injection sites or other sites
- Thrombocytopenia, more than 20% drop in hematocrit following volume replacement treatment compared to baseline

- Signs of plasma leakage (pleural effusion, ascites, hypoproteinemia)

Dengue shock syndrome (DSS) All the above criteria for DHF with evidence of circulatory failure manifested by rapid and weak pulse and narrow pulse pressure (mm Hg) or hypotension for age, cold and clammy skin and restlessness.

Distribution Dengue was initially confined to the east coast of India and caused epidemics (sometimes along with the chikungunya virus). Subsequently, it spread westwards and, in the 1990s, Surat and Delhi had major epidemics with deaths due to DHF and DSS. All four types of dengue virus are present in this country. Occasionally, more than one type has been isolated from the same patient.

Laboratory diagnosis The virus can be isolated in the first week of illness but this is rarely done. The mainstay of diagnosis is the detection of a non-structural viral protein antigen (NS1). This can be detected in the blood up to 7–10 days of onset of disease. Demonstration of circulating IgM antibody provides early diagnosis as it appears within two to five days of onset of illness and persists for one to three months. The IgM ELISA test offers reliable diagnosis. A strip immunochromatographic test for IgM is available for rapid diagnosis. However, the test has to be confirmed by ELISA. The IgG antibodies are detected in a paired serum sample to show rising titres.

Control of dengue is limited to vector control. WHO has approved a vaccine which has been licensed and launched in some South American countries. In India, the vaccine is yet to be launched. Clinical trials of bivalent, trivalent and quadrivalent dengue vaccines are being initiated to study the efficacy and safety of these vaccines before they can be put to use.

Treatment There is no specific treatment for dengue. Supportive management, with cold tepid sponging, paracetamol for fever (Aspirin/NSAIDs like Ibuprofen, etc., should be avoided since they may cause gastritis, vomiting, acidosis, platelet dysfunction and severe bleeding), fluid and electrolyte replacement and platelet infusion when counts are 10,000 and less, should be done. Dengue shock is treated with whole blood transfusion and management of shock.

Zika virus

Zika virus has recently been recognised as a global threat. The virus

belongs to the Flaviviridae group and is transmitted by Aedes mosquito.

It was first isolated in 1947 from a rhesus monkey in the Zika forest in Uganda. Zika is a sylvatic disease that is maintained in mosquito and non-human primates. Outbreaks have occurred in French Polynesia and South Pacific islands. It is associated with neurological complications and the Guillain–Barré syndrome. Diagnosis is by demonstrating the viral RNA by RT-PCR.

Tick-Borne Group

These viruses produce two clinical syndromes: **encephalitis** and **hemorrhagic fevers**.

Tick-borne encephalitis viruses

A number of viruses belonging to the Russian spring summer encephalitis (RSSE) complex cause encephalitis along a wide area of the northern landmass, from Scotland to Siberia. The names given to the disease vary from one area to another depending on variations in the prominent clinical features. RSSE is the most serious form, with high rates of fatality and permanent paralytic sequelae in some survivors. Infection is transmitted by the bite of ixodid ticks. The virus is transmitted transovarially in ticks so that they can act as vectors as well as reservoir hosts. Wild rodents and migrating birds are other reservoirs. Biphasic meningoencephalitis may be transmitted to human beings when they drink the milk of infected goats. Control of infection by the RSSE complex depends on avoiding tick bites. A formalin-inactivated RSSE vaccine has been found useful.

Tick-borne hemorrhagic fevers

Kyasanur Forest disease (KFD)

This is a hemorrhagic fever found in Karnataka (India). In 1957, several dead monkeys were found in the Kyasanur Forest in the Shimoga district of Karnataka and a severe prostrating illness was noticed in some of the villagers in the area. A new arbovirus antigenically related to the RSSE complex was isolated by investigators from the National Institute of Virology (then Virus Research Centre), Pune, from the patients and dead monkeys. It was named the KFD virus after the place where the first isolations were made. An outbreak of KFD was reported in 2015 from

Wayanad and Malappuram districts of Kerala.

Forest birds and small mammals are believed to be the reservoir hosts (Fig. 50.2). Infection is transmitted by the bite of ticks, the principal vector being *Haemaphysalis spinigera*. As infection in monkeys leads to fatal disease, they are not the primary reservoirs but only amplifier hosts. *Haemaphysalis* ticks may act as the reservoir to some extent as transovarial transmission of the virus has been demonstrated in them.

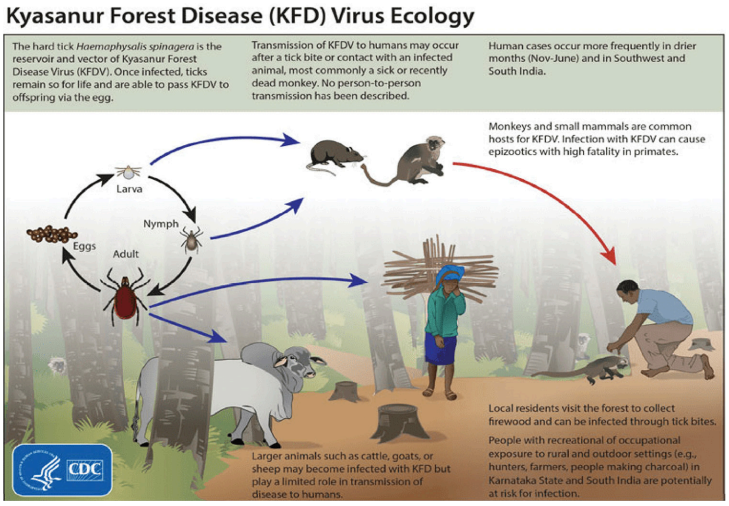

Kyasanur Forest Disease (KFD) Virus Ecology
The hard tick Haemaphysalis spinagera is the reservoir and vector of Kyasanur Forest Disease Virus (KFDV). Once infected, ticks remain so for life and are able to pass KFDV to offspring via the egg.
Transmission of KFDV to humans may occur after a tick bite or contact with an infected animal, most commonly a sick or recently dead monkey. No person-to-person transmission has been described.
Human cases occur more frequently in drier months (Nov-June) and in Southwest and South India.
Monkeys and small mammals are common hosts for KFDV. Infection with KFDV can cause epizootics with high fatality in primates.
Larva
Nymph
Eggs
Adult
Local residents visit the forest to collect firewood and can be infected through tick bites.
People with recreational of occupational exposure to rural and outdoor settings (e.g., hunters, farmers, people making charcoal) in Karnataka State and South India are potentially at risk for infection.
Larger animals such as cattle, goats, or sheep may become infected with KFD but play a limited role in transmission of disease to humans.
CDC

Fig. 50.2 Ecology of Kyasanur Forest disease (*Source:* https://www.insightsonindia.com/2019/03/25/in-news-kyasanur-forest-disease/)

Clinical features KFD has an abrupt onset of fever, headache, conjunctivitis, myalgia and severe prostration. Some cases develop hemorrhages in the skin, mucosa and viscera. The case fatality rate is about 5 per cent. A killed KFD virus vaccine was used in a small field trial and appeared to provide some degree of protection.

History

For many years after its discovery in 1957, the epizootic and epidemic activity of KFD remained confined to the areas contiguous to its original focus in the Sagar, Sorab and Shikarpur taluks of Shimoga district.

Following the felling of a part of an evergreen reserve forest in the area in September 1982, an epizootic and epidemic of KFD occurred in south Kanara. The outbreak, known locally as '**monkey fever**', started with dead monkeys being observed followed by 1,142 human cases with 104 deaths. The ecological disturbance caused by the felling of the virgin forest is believed to have activated a silent enzootic focus of the virus.

Omsk hemorrhagic fever This occurs in Russia and Romania. It is clinically similar to KFD and is caused by a related virus. *Dermacentor* ticks are the vectors.

FILOVIRIDAE

Ebola virus

The virus family Filoviridae includes three genera: *Cuevavirus*, *Marburgvirus*, and *Ebolavirus.*

Within the genus *Ebolavirus*, five species have been identified:

i) Zaire
ii) Bundibugyo
iii) Sudan
iv) Reston
v) Taï Forest

The virus that caused the 2014–2016 West African outbreak belonged to the Zaire Ebola virus species. The Ebola virus is responsible for hemorrhagic fever. It causes an acute illness which is serious and often fatal if untreated. The infection first occurred in a village near the Ebola river, after which the virus is named. The outbreak in 2014–2016 in West Africa spread very rapidly claiming many lives. It spread between countries within Africa, starting in Guinea then moving across land borders to Sierra Leone and Liberia.

Pathogenesis The incubation period is 2 to 21 days. Humans are not infectious until they develop symptoms. The first symptoms are the sudden onset of fever, fatigue, muscle pain, headache and sore throat. This is followed by vomiting, diarrhea, rash, symptoms of impaired kidney and liver function, and in some cases, both internal and external bleeding (e.g., oozing from the gums, blood in the stools). Laboratory findings include low white blood cell and platelet counts and elevated liver enzymes.

Diagnosis The disease can be diagnosed by antigen capture ELISA, RT-PCR and antibody-capture ELISA.

Management of cases must be done under strict containment with standard precautions and personal protective equipment. Health care workers are specifically at risk and hence must strictly adhere to infection control practices.

Treatment Supportive therapy with intravenous fluids to maintain hydration, drugs for blood pressure, and other treatment for specific symptoms make up the standard protocol. Two new antiviral drugs have

been tried and show promising results—regeneron (REGN-EB3) and mAb114. Treatment with these two drugs resulted in much higher overall survival. These two antiviral drugs currently remain in use for patients with confirmed Ebola.

Vaccines WHO released an experimental Ebola vaccine which proved highly protective against the virus in a major trial in Guinea. The vaccine is called **rVSV-ZEBOV.** The current strategy involves vaccinating anyone who's been in contact with an infected person and all the contacts as follows:

Innovative strategy

1. *Pop-up vaccination* This strategy is to vaccinate the population at risk in a protected vaccination site, i.e., health care facility, at a distance from the residence of the contacts.
2. *Targeted geographic vaccination* All listed contacts and their contacts are vaccinated at a protected health care facility.

Another approach is **ring vaccination**:

1. rVSV-ZEBOV is offered to all high-risk persons (contacts and their contacts) and health care workers.
2. Vaccination of persons who can potentially transmit to their contacts (3^{rd} level contacts) forms a barrier around all contacts.

Two other vaccines are (i) 26 vectored glycoprotein/MVA-BN (Ad26.ZEBOV/MVA-BN) and (ii) Ad5-EBOV) vaccine.

[*Source:* WHO Ebola Vaccines Decision framework; April 12, 2019–www.who.int/blueprint/priority-diseases/keyaction/ ebola-vaccine-candidates/en/]

BUNYAVIRIDAE

This family contains over 300 species and is the largest group of arboviruses. The virus is about 100 nm in diameter and has a complex structure with a triple segmented genome of single-stranded RNA.

Most bunyaviruses are **mosquito-borne**. Some are transmitted by **sandflies** (for example, Phlebotomus fever) or **ticks** (Crimean–Congo hemorrhagic fever). Some are established causes of natural disease, including epidemics and epizootics, while others have been isolated only from insect vectors, not associated with any human or animal disease.

Bunyaviruses are so named from the type species *Bunyamwera* virus isolated from mosquitoes in Uganda in 1946.

The family Bunyaviridae contains four genera of medical importance: *Bunyavirus*, *Phlebovirus*, *Nairovirus* and *Hantavirus* (Hantaviruses are not arthropod-borne and so are discussed under rodent-borne infections). A number of viruses are as yet ungrouped.

Genus *Bunyavirus*

The genus contains over 150 species, of which only a few cause human infections. The clinical disease caused is encephalitis, aseptic meningitis and fever. The California encephalitis group of viruses are endemic in the USA. Large epidemics of fever with aseptic meningitis have been caused by the Oropouche virus (member of the Simbu group) in Brazil. The midge *Culicoides paraensia* is the main vector for the Oropouche virus.

Genus *Phlebovirus*

The main members of this genus are the sandfly fever and Rift Valley fever viruses.

Phlebotomus or **sandfly fever**, also known as Pappataci fever and three-day fever, is a self-limiting, non-fatal fever transmitted by the bite of the sandfly *Phlebotomus papatasii*. It occurs along the Mediterranean coast and Central Asia, extending as far east as Pakistan and northwestern India. The virus has been isolated from sandflies and patients in India. No vertebrate host other than humans has been identified.

Rift Valley fever is a mosquito-borne virus that causes enzootic hepatitis in sheep and other domestic animals in Africa. It is named after the Rift Valley, Kenya. Human infections resemble influenza. It has been reported from Egypt, Kenya, Yemen and Saudi Arabia and has caused epidemics and deaths.

Genus *Nairovirus*

The genus is named after the type species Nairobi sheep disease virus. Members of the Crimean Congo hemorrhagic group are the main human pathogens in this genus. The Crimean hemorrhagic fever virus, first isolated in Crimea in 1945, was subsequently found to be identical to the Congo fever virus isolated in 1956 in Congo (Zaire). Hence the name Crimean Congo hemorrhagic fever (CCHF). The disease is endemic in eastern

Europe, Central Asia and many parts of Africa. Cattle, sheep, goats and other domesticated animals act as natural reservoirs. It is transmitted by *Hyalomma* ticks. During the acute phase of the disease, the blood of the patient is highly infectious and direct transmission may occur through contact. A related virus, *Hazara*, has been isolated in Pakistan. It is also widespread in Iran, Iraq and the UAE. The first case of CCHF was reported from Ahmedabad, Gujarat, in 1911 as a hospital outbreak. Subsequently, several cases have been reported from Gujarat, Rajasthan and UP between 2012 and 2015. Antibodies to the CCHF viruses have been detected in human and animal sera from India.

The **Ganjam virus**, isolated from ticks collected from sheep and goats in Orissa, India, is closely related to the Nairobi sheep disease virus. This virus has also been isolated from humans.

REOVIRIDAE

The genus *Orbivirus* of the family Reoviridae contains arthropod-borne viruses which infect animals and humans. They differ from other arboviruses in having double-stranded RNA genomes.

The Colorado tick fever virus, an orbivirus, causes a self-limiteding, mild fever without a rash. It is spread by the wood tick *Dermacentor andersoni*.

The Palyam, Kasba and Vellore viruses belonging to the *Orbivirus* group have been isolated from mosquitoes in India but their pathogenic significance is not known.

UNGROUPED ARBOVIRUSES

A number of arboviruses isolated from insects, animals, birds and human beings have not yet been assigned to any taxonomic group. Examples of ungrouped arboviruses isolated from India are as follows:

Wanowrie virus This was isolated from *Hyalomma* ticks in India and from the brain of a young girl who died after a two-day fever in Sri Lanka. The virus is also present in Iran and Egypt.

Bhanja virus This was isolated from *Haemophysalis* ticks from goats in Ganjam. Human infections with disease and death have been reported from Yugoslavia.

RODENT-BORNE VIRUSES

A similar ecological group is that of the rodent-borne (robo) viruses maintained in nature and transmitted by rodents, and sometimes infecting other species, including humans. Roboviruses, like arboviruses, belong to different taxonomical families, some of them in common with arboviruses.

Genus *Hantavirus*

This virus causes hemorrhagic fever with renal syndrome (HFRS), also known as endemic or epidemic nephrosonephritis.

The disease occurs in two forms: the milder epidemic nephritis (EN) common in Scandinavia and the more serious epidemic hemorrhagic fever (EHF) in the Far East. The clinical picture resembles typhoid, leptospirosis and scrub typhus.

The genus contains at least four species:

i) **Hantaan virus**, which causes the severe HFRS in the Far East, North Asia and Russia.

ii) **Seoul virus,** which causes a milder type of disease and is probably present worldwide.

iii) **Puumala virus,** which is responsible for nephropathia epidemica in northern and eastern Europe.

iv) **Prospect Hill virus,** isolated from voles in the USA, which has not been associated with human illness.

Hantavirus species are natural pathogens of rodents—field mice (*Apodemus agrarius*) and rats (*Rattus rattus* and *R. norvegicus*) in Seoul and voles in Puumala and Prospect Hill. Viremia is present in infected rodents and shed in urine, feces and saliva in high titres. Transmission from rodent to rodent and rodent to humans is primarily by inhalation of the virus contained in dried excreta. Domestic rats appear to be the source of infection in urban cases of HFRS. Demonstration of IgM antibody by ELISA or of rising titres of immune adherence hemagglutinating antibodies in paired sera confirms the diagnosis.

A new syndrome, the **hantavirus pulmonary syndrome,** was identified in southwestern USA in 1993. After a prodrome of fever, malaise, myalgia and gastrointestinal symptoms lasting for 3–4 days, patients develop pulmonary involvement with pulmonary edema. In severe cases, tachypnea,

tachycardia, hypotension and hypoxia lead to death. The disease is caused by a newly identified Hantavirus, the *Sin Nombre* (meaning nameless) virus, which is associated with the deer mouse and other rodents. No arthropod has been linked with the transmission of the virus. Infection appears to be caused by the inhalation of virus aerosols in dried rodent feces.

Key points

- Arboviruses (arthropod-borne viruses) are transmitted by hematophagous insect vectors. They multiply in bloodsucking insects (mosquitoes and ticks) and are transmitted by bite to vertebrate hosts.
- They can be cultivated by intracerebral inoculation of suckling mice (optimal method), primary cell cultures (chick embryo fibroblasts) and continuous cell lines [HeLa, Vero embryonated hen eggs (yolk sac and chorioallantoic membrane)].
- Most arboviruses agglutinate chick erythrocytes by hemagglutinin antigens; inhibition of this by specific antibody is used for diagnosis.
- Complement fixing and neutralising antibodies have also been detected in serological studies.
- Arboviruses are generally labile, being inactivated at room temperature and by bile salts, ether and other lipid solvents.
- Arboviruses cause the following syndromes with varying degrees of severity (sometimes, subclinical infections):
    - Fever with or without rash and arthralgia (dengue types 1–4, chikungunya, O'nyong-nyong, Ross River, West Nile, sandfly fever, Rift Valley fever, Colorado tick fever)
    - Encephalitis (equine encephalitis, Japanese B encephalitis, West Nile fever)
    - Hemorrhagic fevers (chikungunya, dengue types 1–4, Kyasanur Forest disease, Crimean Congo hemorrhagic fever)
    - Yellow fever affecting the liver
- Japanese encephalitis is a serious illness, and there have been several outbreaks in India. It is transmitted by *Culex* mosquitoes and amplifier hosts include birds, cattle, buffaloes and pigs.
- Yellow fever is a hemorrhagic fever of humans and primates caused by the yellow fever virus—a member of Flaviviridae. It is confined to certain areas of the world (Central Africa and parts of South and Central America). Active immunisation with the 17D strain of live, attenuated yellow fever vaccine is required by travellers visiting endemic regions.
- Dengue fever is an acute febrile disease caused by the dengue fever virus (types 1–4). The virus is transmitted by the *Aedes* mosquito. Primary infection results in moderately severe

febrile illness. Subsequent infection with a different serotype may induce severe hemorrhagic fever and dengue shock syndrome. Laboratory diagnosis is usually by demonstrating antibodies, detection of viral RNA, and rarely, by the isolation of the virus.

- The chikungunya virus has caused epidemics in India. Humans are the host, and *Aedes aegypti* mosquitoes the vectors.
- Kyasanur Forest disease is a hemorrhagic fever from the Shimoga district in Karnataka which has now spread to other areas within and adjoining the state. The tick *Haemaphysalis spinigera* is the vector for the causative organism.
- The genus *Hantavirus* consists of at least four serogroups. Wild rodents (species of mice, rats, voles) are the hosts. Transmission to humans is through inhalation of infectious aerosols from rodent excreta. Acute hantaviral infections are characterised either by acute renal failure (nephritis) and hemorrhage or a syndrome of acute non-cardiogenic pulmonary edema.

Questions

Essays

1. How are arboviruses classified? List the diseases caused by them in India. Describe the laboratory diagnosis of any one virus.
2. Define arboviruses. Discuss mosquito-borne arboviral infections in India.
3. Describe the epidemiology and diagnosis of dengue fever.
4. Describe the epidemiology, laboratory diagnosis and prevention of Japanese encephalitis.

Short Answers

1. Tick-borne arboviruses
2. Flowchart for the transmission of Japanese encephalitis
3. Vectors and the viral infections transmitted by them
4. Dengue hemorrhagic fever
5. Yellow fever
6. Serological tests for the diagnosis of arboviral infections
7. Kyasanur Forest disease
8. List of rodent-borne viruses and epidemiology of Hantaviruses

Part IV: Virology

CHAPTER 51

Rhabdovirus and Other Viral and Prion Diseases

Chapter navigation

- Rhabdovirus (Rabies virus)
- Slow virus disease
- Prion disease

INTRODUCTION

The family Rhabdoviridae contains viruses that infect mammals, reptiles, birds, fish, insects and plants. Some members multiply in vertebrates and arthropods. These are **bullet-shaped, enveloped viruses** with a single-stranded RNA genome.

Rhabdoviruses infecting mammals belong to **two genera**:

- *Vesiculovirus,* comprising vesicular stomatitis virus and related viruses (which do not infect humans) and
- *Lyssavirus* (*lyssa*, meaning rage, a synonym for rabies), comprising rabies and related viruses.

Clinical Case 1

A five-year-old boy was bitten by a rabid dog and presented to the Emergency department with multiple scratches on the upper arms and neck. His parents stated that the dog was a stray that lived in their locality (so it could be observed). The child was given local wound toileting, followed by the application of povidone-iodine. The

wound was left open. He was given tetanus toxoid (as his booster was due) and Vero cell culture vaccine was started. He completed the course (0, 3, 7, 14 and 28 days post-exposure). The dog died two days later. Its brain was sent to the veterinary lab and tested positive for Negri bodies on histopathology.

Clinical Case 2

A 30-year-old woman was brought to a referral hospital from a rural area where she had been admitted with fever, anorexia and vomiting for the previous four days followed by delirium and hallucination and severe spasms on drinking fluids. Her relatives informed the doctor that she had been bitten by a stray dog about three months back. No medical help had been sought at the time, but wound care and local treatment were done. Following admission, she was started on IV fluids and other supportive care. The next day, she developed severe spasms in the pharyngeal muscles. This spasm increased in intensity and distribution leading to generalised convulsions despite anticonvulsion therapy. She died during one of these attacks due to respiratory failure. Post-mortem was conducted and the brain tissue was found positive for rabies virus antigen by direct fluorescent antibody test.

RABIES

Rabies has been recognised since ancient times as a disease transmitted to humans and animals by the bite of 'mad dogs'. The name 'rabies' comes from the Latin word *rabidus*, meaning mad, derived from the Sanskrit root *rabhas*, for frenzy. The disease in human beings is called **hydrophobia** because the patient exhibits a fear of water.

Pasteur (in 1881) established that the rabies virus was present in the brain of infected animals. By serial intracerebral passage in rabbits, he obtained the fixed virus that could render dogs immune by a series of injections of fixed virus. This vaccine was prepared by drying pieces of spinal cord from rabbits infected with the fixed virus. In July 1885, **Joseph Meister**, a nine-year-old boy, severely bitten by a rabid dog and in grave risk of developing rabies, was given a course of 13 inoculations of the infected cord vaccine by Pasteur. The boy survived. This dramatic event was a milestone in the development of medicine.

Rabies Virus

Morphology

The rabies virus is bullet shaped, 180 × 75 nm in size, with one end rounded or conical and the other planar or concave. The lipoprotein envelope carries

knob-like spikes, composed of glycoprotein G. Spikes may be released from the envelope by treatment with lipid solvents or detergents. Beneath the envelope is the membrane or matrix (M) protein. The membrane may project outwards from the planar end of some virions, forming a bleb. The core of the virion consists of helically arranged ribonucleoprotein (Fig. 51.1). The genome is an unsegmented, linear, negative sense RNA. RNA-dependent RNA transcriptase and some structural proteins are present in the nucleocapsid.

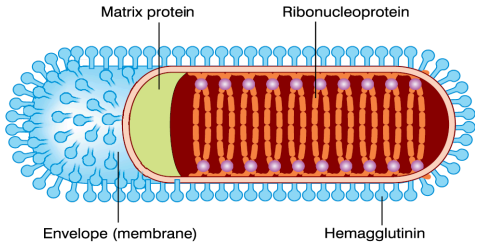


Fig. 51.1 Bullet-shaped rabies virus with its structural elements

Antigenic Properties

1. **Glycoprotein** The surface spikes are composed of glycoprotein G, which is important in pathogenesis, virulence and immunity. The important properties are as follows:

    - It mediates the binding of the virus to acetylcholine receptors in neural tissues.
    - It induces hemagglutination inhibiting (HI) antibodies. Rabies virus possesses hemagglutinating activity, optimally seen with goose erythrocytes at 0–4°C and a pH of 6.2.
    - It induces neutralising antibodies (protective antibodies).
    - It stimulates cytotoxic T cell immunity.
    - Purified glycoprotein may act as a safe and effective subunit vaccine.

2. **Nucleoprotein** This is a nucleocapsid protein, with the following properties:

    - It induces complement-fixing antibodies, which are not protective.

- The antigen is group-specific and cross-reactions are seen with some rabies-related viruses.
- The antiserum prepared against the nucleocapsid antigen is used in diagnostic immunofluorescence tests.

Other antigens identified include two membrane proteins, glycolipid and RNA-dependent RNA polymerase.

Susceptibility

The virus is inactivated by ethanol, iodine preparations, quaternary ammonium compounds, soap, detergents and lipid solvents such as ether, chloroform and acetone. It is inactivated by phenol, formalin, beta-propiolactone, ultraviolet irradiation and sunlight. Thermal inactivation occurs in one hour at 50°C and five minutes at 60°C. It dies at room temperature but can survive for weeks when stabilised by 50% glycerol. It survives at 4°C for weeks. It can be preserved at –70°C or by lyophilisation. For storage in dry ice, the virus has to be sealed in vials as it is inactivated on exposure to CO_2.

Host Range and Cultivation

Animals All mammals are susceptible to rabies infection, though differences in susceptibility exist between species. Cattle, cats and foxes are highly susceptible, whereas skunks, opossums and fowl are relatively resistant. Humans and dogs occupy an intermediate position.

Rabies virus has been classified into two types, street and fixed virus, based on the naturally infective strain and laboratory-maintained strain (Table 51.1).

Table 51.1 Differences between the types of rabies virus

Feature	Street virus	Fixed virus
Isolation	Natural human or animal infection	After several serial intracerebral passages in rabbits
Infectivity and fatal encephalitis	Inoculation via any route	More neurotropic, though it is much less infective by other routes

Incubation period	Long and variable incubation period of about 1–12 weeks	Short and fixed incubation period of 6–7 days
Negri body demonstration	Can be demonstrated in the brain of animals dying of street virus infection	Usually not demonstrable
Vaccine production	Not used	Used

Pathogenicity

Transmission **Human infection** is usually caused by the bite of rabid dogs or other rabid animals. The virus present in the saliva of the animal is deposited in the wound. Rarely, infection may also occur following non-bite exposures, aerosols, transplantation of cornea or other virus-infected tissues. Humans appear to possess a high degree of natural resistance to rabies. Rabies antibodies demonstrated in veterinarians without any history of antirabic vaccination suggests that **inapparent infection** may occur.

Incubation period in humans It is usually 1–3 months, though it may be as short as seven days or as long as three years. The incubation period is usually short in persons bitten on the face or head and long in those bitten on the legs. This may be related to the distance the virus has to travel to reach the brain. The incubation period is generally shorter in children than in adults (*Clinical cases* 1 and 2).

The virus appears to multiply in the muscles, connective tissue or nerves at the site of deposition for 48–72 hours. The virus spreads via the peripheral nerves to the brainstem or spinal cord at a rate of 3 mm/hour via retrograde axonal transport (Fig. 51.2a and b).

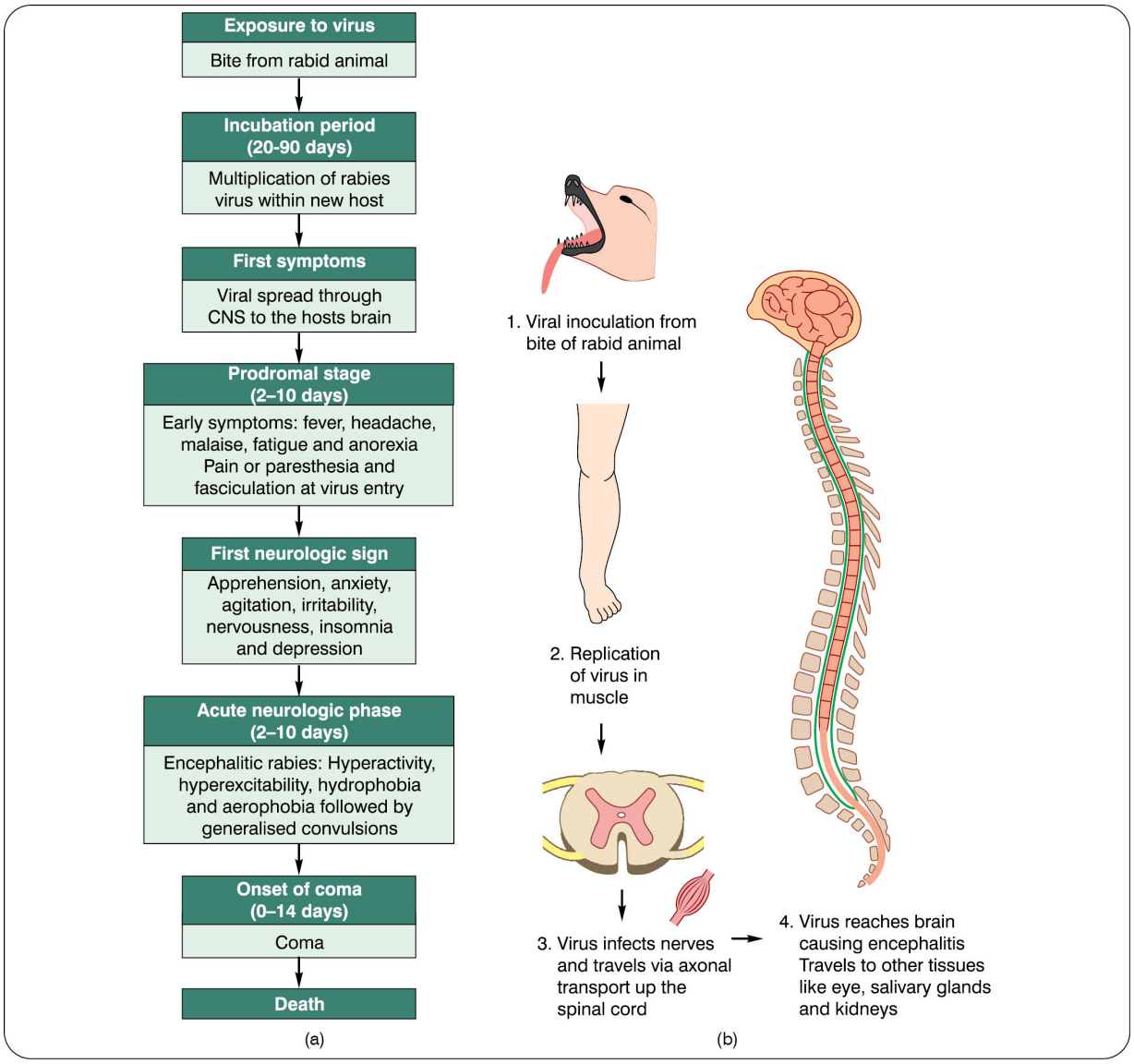

Exposure to virus
Bite from rabid animal
Incubation period (20-90 days)
Multiplication of rabies virus within new host
First symptoms
Viral spread through CNS to the hosts brain
Prodromal stage (2–10 days)
Early symptoms: fever, headache, malaise, fatigue and anorexia Pain or paresthesia and fasciculation at virus entry
First neurologic sign
Apprehension, anxiety, agitation, irritability, nervousness, insomnia and depression
Acute neurologic phase (2–10 days)
Encephalitic rabies: Hyperactivity, hyperexcitability, hydrophobia and aerophobia followed by generalised convulsions
Onset of coma (0–14 days)
Coma
Death
(a)
1. Viral inoculation from bite of rabid animal
2. Replication of virus in muscle
3. Virus infects nerves and travels via axonal transport up the spinal cord
4. Virus reaches brain causing encephalitis Travels to other tissues like eye, salivary glands and kidneys
(b)

Fig. 51.2 (a) Flowchart depicting the pathogenesis and sequence of events in rabies and (b) diagrammatic representation of pathogenesis

It disseminates throughout the central nervous system along neuroanatomical connections and selectively injures neuronal processes (axons and dendrites) without producing neuronal death**. Histopathologic changes in encephalitis** show perivascular cuffing due to the accumulation of inflammatory cell infiltrates, including lymphocytes and polymorphonuclear leucocytes (PMNs)(Fig. 51.3).

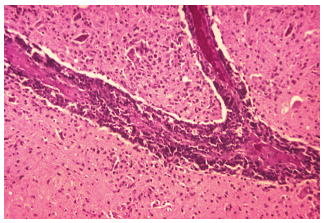


Fig. 51.3 Histopathologic changes associated with rabies encephalitis shows perivascular cuffing by inflammatory cells like lymphocytes and polymorphonuclear leucocytes (*Source:* Public Health Image Library, ID 4069 / Dr. Daniel P. Perl / CDC)

The virus also **multiplies in the salivary glands** and is shed in saliva. It ultimately reaches virtually every tissue in the body, though centrifugal dissemination may be interrupted at any stage by death. The virus is almost invariably present in the **cornea and facial skin of patients, which provides a method for the ante-mortem diagnosis of human rabies.**

Clinical Stages

The course of the disease in humans can be classified into four stages:

1. ***Prodrome*** Onset is marked by prodromal symptoms such as fever, headache, malaise, fatigue and anorexia. An early symptom is often a neuritic type of pain or paraesthesia and fasciculation at the site of virus entry. Apprehension, anxiety, agitation, irritability, nervousness, insomnia and depression characterise the prodromal phase, which usually lasts 2–10 days. Excessive libido, priapism and spontaneous ejaculation may occur rarely.
2. ***Encephalitic rabies*** The acute neurological phase usually begins with **hyperactivity**, which is characteristically intermittent with bouts of bizarre behaviour, agitation or seizures appearing between apparently normal periods in 2–10 days. Such hyperactivity may be spontaneous or precipitated by external stimuli. The pathognomonic feature is difficulty in drinking together with intense thirst. Patients may be able to swallow dry solids but not liquids. Attempts to drink bring on such painful spasms of the pharynx and larynx, producing choking or gagging, that patients develop a dread of even the sight or sound of water (hydrophobia). **Cardinal features are hyperexcitability, hydrophobia and aerophobia followed by generalised convulsions**.

3. ***Paralytic rabies*** In about 20% of cases, encephalitic rabies is absent. Muscle weakness is predominant with flaccid weakness starting at the site of the bite which spreads to produce quadriparesis and facial weakness in 2–10 days. These patients may survive a little longer than the encephalitic group. But ultimately, multiorgan failure occurs, followed by death. Paralytic rabies may be confused with Guillain–Barré syndrome.
4. ***Coma and death*** Some patients may enter into a comatose state. **Death** usually occurs within 1–14 days due to respiratory arrest during convulsions.

Rabies in dogs (or other animals)

- **In dogs**, the incubation period is usually 3–6 weeks but it may range from 10 days to a year. The initial signs are an alert, troubled air and a change in disposition with restlessness, snapping at imaginary objects, licking or gnawing at the site of the bite. After 2–3 days of this prodromal stage, the disease develops into either the furious or dumb type of rabies.
- In **furious rabies,** which is much more common, the dog runs amok, biting without provocation and indiscriminately. The lower jaw droops and saliva drips uncontrollably from the mouth. Paralysis, convulsions and death follow.
- **Dumb rabies,** the second type is the paralytic form in which the animal lies huddled, unable to feed. The dog may not bite but attempts to feed it are dangerous. About 60 per cent of rabid dogs shed the virus in saliva. Rabid dogs usually die in 3–5 days.

Epidemiology

Direct person-to-person transmission of rabies has not been recorded, though the virus is present in the saliva of patients. Therefore, there is no danger in examining or nursing hydrophobia patients provided suitable precautions are taken.

Two epidemiological types of rabies exist: **urban,** transmitted by domestic animals like dogs and cats; and **sylvatic,** involving animals in the wild, such as jackals, wolves, foxes, mongooses, skunks and bats.

The **primary source** of the rabies virus in nature seems to be mustelids and viverrids. The virus survives in this reservoir population by achieving a state of latency. From here, foxes, wolves and jackals acquire the infection and the disease spreads to dogs and other domestic animals.

Another natural cycle of rabies concerns bats. **Vampire bats** may shed the rabies virus as symptomless carriers over a period of several months. Rabies also occurs in **insectivorous and frugivorous bats**. Infection in insectivorous bats is symptomatic, while frugivorous bats become asymptomatic carriers. Humans may be infected by aerosols if they enter caves colonised by infected bats.

Rabies has been eliminated from islands like Britain and Japan by rigid quarantine. Australia, which has no native mustelid or viverrid population, has no cases of rabies. **Rabies is endemic in India**. It has been estimated that more than 30,000 people die of rabies in India every year and more than 700,000 receive antirabies vaccine. In India, antirabies treatment is to be considered following the bite of any animal except rats. Human rabies can be checked by the control of rabies in domestic animals, by registration, licensing and vaccination of pets and culling of stray animals. With the dog population in India estimated to be over 16 million, the problem is immense. National Rabies Control Programme (NRCP) has both human and animal health components. The human health component is being implemented across the country.

Global containment

WHO, the World Organisation for Animal Health (OIE), the Food and Agriculture Organization of the United Nations (FAO) and the Global Alliance for Rabies Control (GARC) have established a global 'United Against Rabies' collaboration to provide a common strategy to achieve a target of zero human rabies deaths by 2030.

Laboratory Diagnosis

In humans

Routine laboratory findings are often normal. CSF shows mild mononuclear cell pleocytosis with slightly elevated protein. CT and MRI are variable and non-specific.

Specific confirmatory diagnosis

1. **Specimen** The specimens tested are corneal smears and skin biopsy (from the face or nape of the neck) or saliva ante-mortem and brain post-mortem.

2. **Direct microscopy**

Ante-mortem

- **Skin biopsy** (hairy skin from the nape of the neck) or fresh saliva are subjected to **direct fluorescent antibody testing (DFA)** using monoclonal antibodies tagged with fluorescein isothiocyanate as the method of choice. Immunoperoxidase staining can be used to detect antigen in tissues. Corneal impression smears are rarely used as the sensitivity is very low.
- **Antibody demonstration** Rabies antibodies in the CSF suggest rabies encephalitis. High-titre antibodies are present in the CSF in rabies during infection but not after immunisation. Demonstration of antibodies by ELISA has been used for determining the antibody response in laboratory personnel who are exposed to the rabies virus.
- RT-PCR with genetic sequencing is highly sensitive and can detect the viral RNA from CSF, saliva, skin and brain tissue (post-mortem).

Post-mortem Diagnosis may be made by the demonstration of **Negri bodies** in the brain tissue from the pyramidal cells of Ammon's horn, and the Purkinje cells of the cerebellum. They may be absent in about 20% of cases.

3. **Isolation**

Cultivation of rabies virus This is done only in reference laboratories with biosafety level III facilities.

- **Chick embryos** The rabies virus can be grown in chick embryos. The usual mode of inoculation is into the yolk sac. Serial propagation in chick embryos has led to the development of attenuated **vaccine strains like Flury and Kelev**.

- **Tissue culture** The rabies virus can grow in several primary and continuous cell cultures.

  A more rapid and sensitive method is the isolation of the virus in tissue culture cell lines (WI 38, BHK 21, CER). Cytopathic effect is minimal, hence immunofluorescence is used to detect replication in the cell line. A positive IF test can be obtained as early as 2–4 days after inoculation. The fixed virus strains are adapted for growth in **human diploid cell** and **Vero cell cultures** which are used for the production of vaccines.
- Chick embryo fibroblast, and porcine or hamster kidney cells also support the rabies virus but cytopathic effects are not apparent and the yield of virus is low.
- **Animal inoculation** Isolation of the virus by intracerebral inoculation in mice can be attempted from the brain, CSF, saliva and urine (biological test). Chances of isolation are greater early in the disease. The inoculated mice are examined for signs of illness and their brains are examined at death or at 28 days post-inoculation for Negri bodies, or by immunofluorescence. Neutralising antibodies can be detected.

Laboratory confirmation of rabies in animals

It is of great importance in assessing the risk of infection and deciding post-exposure treatment. The whole carcass or the severed head of the animal suspected to have died of rabies may be sent to the reference laboratory. Alternatively, the brain may be removed carefully and two portions—one in 50% glycerol saline and the other in Zenker's fixative—sent for biological test and microscopy respectively. The portion of brain sent should include the hippocampus and cerebellum since **Negri bodies** are most abundant there. The following tests are done in the laboratory:

1. ***Demonstration of inclusion bodies (Negri bodies)*** This is still the most commonly used method in addition to immunofluorescence. Impression smears of the brain are stained by **Seller's technique** (basic fuchsin and methylene blue in methanol). Negri bodies are seen as intracytoplasmic, round or oval, purplish-pink structures with characteristic basophilic inner granules. Negri bodies vary in size, 3–

27 ∝m (Fig. 51.4). **Failure to find Negri bodies does not exclude the diagnosis of rabies.**

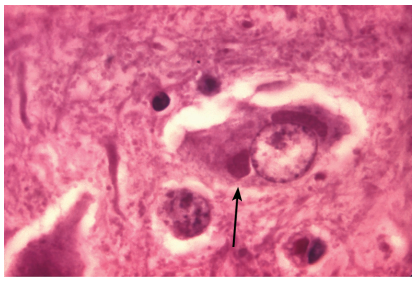


Fig. 51.4 Seller's stain showing intracytoplasmic inclusion body (Negri body) found most frequently in the pyramidal cells of Ammon's horn, and the Purkinje cells of the cerebellum (*Source:* Public Health Image Library, ID 3372 / Dr. Daniel P. Perl / CDC)

2. ***Demonstration of rabies virus antigen by immunofluorescence***
Examination of the salivary glands by immunofluorescence is useful. It may indicate whether the animal was shedding the virus in saliva.

Rabies virus isolation and other laboratory procedures are done in reference laboratories equipped with biosafety cabinets and expertise to handle rabid tissues. National Centre for Disease Control (NCDC) in Delhi is the designated reference laboratory in the country.

Prophylaxis

Pre-exposure Rabies immunoprophylaxis is mandatory for all pet animals.

In humans, immunisation is recommended for persons at high risk such as veterinarians and dog handlers. Cell culture vaccines, which are free from serious complications, have made pre-exposure immunisation in humans safe and feasible. Pre-exposure vaccination is administered as one full dose of vaccine intramuscularly or 0.1 mL intra-dermally on days 0, 7 and either day 21 or 28. Laboratory staff and others at high continuing risk of exposure should have their neutralising antibody titres checked every 6 months during the initial two-year period after the primary vaccination. If it is less than 0.5 IU/mL, a booster dose of vaccine should be given (National Guidelines on Rabies Prophylaxis).

Post-exposure management Specific prophylaxis is generally used after exposure to infection and is therefore called **antirabies treatment.** This

consists of local treatment, active immunisation with antirabies vaccines and passive immunisation with antirabies serum as follows:

i) Local treatment Animal bites deposit the virus in the wound. Hence the wound should be scrubbed immediately with soap and water. This is a very important step in the prevention of rabies as **soap destroys the virus effectively**. The wound is then treated with quaternary ammonium compounds (such as cetavlon), tincture or aqueous solution of iodine, or alcohol (40–70%). In severe wounds, antirabies serum may be applied topically and infiltrated around the wound. It is advisable to **postpone suturing the wound**. Anti-tetanus measures and antibiotics to prevent sepsis may be used as necessary.

ii) Active immunisation (antirabies vaccines) Active immunisation is currently done using the following vaccines:

- **Tissue culture vaccines**
  - **Human diploid cell (HDC)** vaccine was the first cell culture vaccine and was developed by Koprowski, Wiktor and Plotkin. It is a purified and concentrated preparation of fixed rabies virus (Pitman–Moore strain) grown on human diploid cells (WI 38 or MRC 5) and inactivated with beta-propiolactone or tri-n-butyl phosphate. It is highly antigenic and free from serious side effects. Its only disadvantage is its high cost.
  - Other equally effective and more economical vaccines have been developed, like the continuous cell culture vaccines grown on the Vero cell line derived from the monkey kidneys.

  In India, the following rabies vaccines are available:

  i) Purified Vero cell rabies vaccine (PVRV)
  ii) Chromatographically purified rabies vaccine (CPRV)
  iii) Human diploid cell vaccine (HDC)
  iv) Purified chick embryo cell vaccine (PCECV)
  v) Purified duck embryo vaccine (PDEV)
- **Subunit vaccine** The glycoprotein subunit on the virus surface, which is the protective antigen, has been cloned and

recombinant vaccines produced. They are still in the experimental stage.

iii) Passive immunisation with antirabies serum *Passive immunisation* of rabies is done by the administration *of* human rabies immunoglobulin (HRIG) pooled *from* the sera *of* immunised human donors. In some countries, *rabies* immunoglobulin derived from horses is sometimes used [equine rabies immunoglobulin (ERIG)]. However, due to adverse effects of type 3 hypersensitivity, equine rabies immunoglobulin is no longer preferred.

Vaccines NOT used for human subjects

- **Neural vaccines** These are suspensions of nervous tissues of animals infected with the fixed rabies virus. Neural vaccines are unsatisfactory as they are poor immunogens, containing mostly nucleocapsid antigen. They may contain infectious agents and hence may be encephalitogenic. Neural vaccines have been abandoned now in most parts of the world since tissue culture vaccines are available at an affordable cost.

Vaccination schedules Antirabic vaccine should be administered when a person has been bitten, scratched or licked by an animal that is rabid or cannot be apprehended. When the animal can be observed, it should not be killed and should be observed for 10 days. This observation period is recommended because the virus may be present in saliva 3–4 days before the onset of symptoms and the animal usually dies within 5–6 days of developing the disease. If the animal remains healthy after this period, there is no risk of rabies and vaccination, if already started, may be discontinued. A rare possibility of the carrier state in dogs cannot be ruled out.

In cases where the vaccine is started and the animal kept under observation, an alternative recommendation is to stop treatment after five days. The animal is observed for a further five days and the vaccine is started again if the animal becomes ill or dies during this period.

The WHO guidelines on **post-exposure prophylaxis** are based on the risk category to which the patient belongs (Table 51.2). All three cell culture vaccines available in India (HDC, PCECV and PVRV) have the same dosage schedule, which is the same for both adults and children.

Table 51.2 Type of contact, exposure and recommended post-exposure prophylaxis

Category	Type of contact with a suspect or confirmed rabid domestic or wild[a] animal, or animal unavailable for testing	Type of exposure	Recommended post-exposure prophylaxis
I	Touching or feeding of animals Licks on intact skin	None	None, if reliable case history is available
II	Nibbling of uncovered skin Minor scratches or abrasions without bleeding	Minor	Administer vaccine immediately[b] Stop treatment if animal remains healthy throughout an observation period of 10 days[c] or if animal is proven to be negative for rabies by a reliable laboratory, using appropriate diagnostic techniques
III	Single or multiple transdermal bites or scratches, licks on broken skin Contamination of mucous membrane with saliva (i.e., licks) Exposures to bats[d]	Severe	Administer rabies immunoglobulin and vaccine immediately Stop treatment if animal remains healthy throughout an observation period of 10 days or if animal is found to be negative for rabies by a reliable laboratory using appropriate diagnostic techniques

[a]Exposure to rodents, rabbits and hares seldom, if ever, requires specific anti-rabies post-exposure prophylaxis.
[b]If an apparently healthy dog or cat in/or from a low-risk area is placed under observation, the situation may warrant delaying initiation of treatment.
[c]This observation period applies only to dogs and cats. Except in the case of threatened or endangered species, other domestic and wild animals suspected as rabid should be humanely killed and their tissues examined for the presence of rabies antigen using appropriate laboratory techniques.
[d]Post-exposure prophylaxis should be considered when contact between a human and a bat has occurred, unless the exposed person can rule out a bite or scratch, or exposure to a mucous membrane.

Post-exposure prophylaxis (by vaccination) This schedule is recommended by the Govt. of India in the National Guidelines on Rabies Prophylaxis on post-exposure regimen [updated **Thai Red Cross Schedule** (2-2-2-0-2)]. It involves the injection of 0.1 mL of reconstituted vaccine per intradermal site and on two sites per visit (one on each deltoid area, an inch above the insertion of deltoid muscle) on days 0, 3, 7 and 28. Day 0 is the date of first dose administration of antirabies vaccine and may not be the date of rabies exposure/animal bite.

This course is expected to give protection for at least five years, during which period, any further exposure may need only one or two booster doses (on days 0, 3), depending on the degree of risk. After five years, it is advisable to give a full five-injection course on exposure to infection.

The vaccine is to be given IM or SC in the deltoid region, or in children, on the anterolateral aspect of the thigh. Gluteal injections are to be avoided as they have been found to be less immunogenic.

Passive immunisation (immunoglobulins) This can be provided by:

- **Equine rabies immunoglobulin (ERIG)** Antirabic serum is manufactured by hyperimmunisation of horses. Crude equine antirabies serum is not to be used as it is liable to induce anaphylactic reactions. Purified ERIG is much safer, though not completely free from risk.
- **Human rabies immunoglobulin (HRIG) Though limited in availability and more expensive,** HRIG is preferred over ERIG. It is free from the danger of sensitisation but should also be free from HIV and hepatitis viruses.

Vaccine failures are known with neural vaccines. In view of the safety of the cell culture vaccine, it would be advisable to recommend the vaccine even when there is the slightest risk of exposure to rabies.

Vaccine for animals Antirabies immunisation in animals is to be done as pre-exposure prophylaxis.

Treatment

Until recently, rabies was considered to be invariably fatal and no serious attempt at treatment was made, apart from sedation. It has now been demonstrated that there can be complete recovery from established rabies with intensive supportive care and management of complications. **No specific antirabies agent is available**.

SLOW VIRUS DISEASES

The term 'slow virus disease' is applied to a group of infections in animals and human beings, characterised by a **very long incubation period** and a **slow but relentless course**, terminating fatally just as is seen in rabies. The concept of 'slow infection' was originally proposed by Sigurdsson (1954), a veterinary pathologist, for slowly progressing infections of sheep, such as scrapie, visna and maedi. The recognition in recent years that some chronic degenerative neurological diseases of human beings may have a similar pathogenesis has led to considerable interest in this concept.

Characteristics of slow virus infections

- Incubation periods ranging from months to years
- Course of illness lasting for months or years, with remissions and exacerbations
- Predilection for the involvement of the central nervous system
- Absence of immune response or an immune response that does not arrest the disease but may actually contribute to pathogenesis
- Genetic predisposition
- Invariable fatal termination

Slow virus diseases may be classified into those caused by (a) conventional virus, (b) prions and (c) slow viral diseases of animals.

- **Conventional virus** consists of two unrelated CNS diseases of human beings: subacute sclerosing panencephalitis (SSPE) and progressive multifocal leucoencephalopathy (PML).
- **Prion disease** of the central nervous system includes Kuru and Creutzfeldt–Jakob disease, collectively known as the **subacute spongiform viral encephalopathies (SSVE),** scrapie and mink encephalopathy.

- Slow viral infections of animals are composed of slowly progressive infections of sheep, caused by serologically related, non-oncogenic retroviruses called lentiviruses (from the Latin *lentus*, meaning slow).

1. Slow Viral Diseases Caused by Conventional Viruses

- **Subacute sclerosing panencephalitis (SSPE)** is seen in young adolescents. It is a very rare delayed **sequel to infection with the measles virus**. The disease sets in many years after the initial infection and is characterised by progressive deterioration of mental and motor functions. Death occurs 1–3 years after the onset of symptoms. Brain cells from patients show serological and electron microscopic evidence of measles virus infection. The virus cannot be isolated in routine cultures, but only by co-cultivation of infected brain cells with susceptible cells of non--neural origin. Measles virus strains isolated from SSPE are defective. Patients show very high levels of measles virus antibody in their serum. The antibody is regularly found in CSF and is pathognomonic. Cellular immune response to measles virus is absent in SSPE.
- **Progressive multifocal leucoencephalopathy (PML)** is a rare subacute demyelinating disease seen in elderly persons whose immune process is impaired as a result of malignancy or immunosuppression. There is progressive deterioration of motor function, vision and speech. Death occurs in 3–4 months. Human **polyomavirus** has been demonstrated by electron microscopy and cultured from brain biopsies of patients.

2. Prion Diseases

Prions are protein-containing particles that are infectious in nature. They are devoid of DNA and RNA. They are unusually **resistant to physical and chemical agents such as heat, irradiation and formalin**. They can be

transmitted to experimental animals by parenteral and oral challenge. Stanley B. Prusiner gave the name **prion** to these **proteinaceous infectious agents**. He was awarded the Nobel Prize for Medicine in 1997 for his pioneering work on prions.

The pathogenic mechanism appears to be proliferation of an **abnormal prion protein (scrapie) (PrP^{sc})** which is derived from the normal cellular prion protein PrP^{c}. The accumulation of PrP^{sc} in the central nervous system as diffuse deposits and in the form of plaques disrupts the architecture and function of the brain and causes cell death, leading to the disease.

The human prion-mediated diseases are called transmissible spongiform encephalopathies (TSE) or **subacute spongiform viral encephalopathies.** They are chronic progressive degenerative diseases of the CNS. The pathology consists of progressive vacuolation in the dendritic and axonal processes of the neurons and extensive astroglial hypertrophy and proliferation, culminating in spongiform degeneration in the grey matter. There is no sign of any inflammatory or immune response.

Human prion diseases

- **Creutzfeldt–Jakob disease (CJD**) This is subacute, presenile encephalopathy, with progressive incoordination and dementia, ending fatally in about a year. Both sporadic and inherited forms of the disease have been seen. Iatrogenic CJD has occurred after corneal transplantation and injection of the pituitary growth hormone, presumably from donors who had the infection.
- **Variant CJD** The appearance of a new variant of CJD affecting younger persons (below 45 years) in Britain in 1996 raised fears of infection through eating beef affected by bovine spongiform encephalopathy (BSE). This aroused a panic reaction about the export of British beef, and many thousands of British cattle had to be slaughtered before the anxiety was allayed.
- **Gerstmann–Sträussler–Scheinker (GSS) syndrome and fatal familial insomnia** These are two variants of CJD. The familial form of CJD is rare. These are caused by the inheritance of mutations of the *PrP* gene.

- **Kuru** Identified in 1957, Kuru (meaning tremor) was a mysterious disease seen only in the Fore tribe inhabiting the eastern highlands of New Guinea. The disease had an incubation period of 5–10 years and led to progressive cerebellar ataxia and tremors, ending fatally in 3–6 months. The infection is believed to have been introduced through cannibalism and maintained by the tribal custom of eating the dead bodies of relatives after death as a part of the ritual. The disease disappeared following the abolition of cannibalism in New Guinea. **Carlton Gajdusek** was awarded the Nobel Prize for Medicine in 1976 for his important contributions to the study of Kuru.

Prion diseases of animals

Scrapie is the prototype prion disease. It has been known as a natural disease of sheep for two centuries. Transmission occurs vertically, from ewe to lamb, and less often, by contact. **Mink encephalopathy** is a scrapie-like disease of mink. It is believed to have spread to mink when they fed on scrapie-infected sheep meat. **Bovine spongiform encephalopathy** (BSE, '**mad cow disease**') has been enzootic in Britain since 1986. The infection is presumed to have spread to cattle by the practice of feeding them with scrapie-infected meat.

Laboratory diagnosis of prion diseases

CSF is usually normal, but may show protein elevation.

CT may be normal or may show cortical atrophy. MRI is more useful in differentiating CJD from other neurological diseases.

The only specific diagnosis of CJD and other human prion disease is by **measuring PrP^{sc}.** This is done by proteolysis, which generates the PrP, which is detected by immunoassay after denaturation. Sequencing of the *PRNP* gene to detect the wild type gene sequence or mutations helps in diagnosing CJD or familial prion disease respectively.

Slow Viral Infections of Animals

- **Visna** is a demyelinating disease of sheep. The disease has an incubation period of about two years. It has an insidious onset

with pareses, progressing to total paralysis and death.

- **Maedi** (progressive pneumonia) is a slowly progressive, fatal hemorrhagic pneumonia of sheep, with an incubation period of 2–3 years. It is classified as one of the lentivirus groups.

Key points

- A bacteriophage is a type of virus that specifically infects bacteria and fungi.
- Bacteriophages exhibit two different types of life cycles:
  - **Lytic** (virulent) cycle, where intracellular multiplication of the phage ends in the lysis of the host bacterium and the release of progeny virions.
  - **Lysogenic** (temperate) cycle, where phage DNA becomes incorporated within the bacterial genome and then replicates synchronously with it, causing no harm to the host cell.
- The lytic cycle replication allows the bacteriophage to proceed through the stages of adsorption (attachment to the host cell), penetration of the nucleic acid into the cell, synthesis of phage components, assembly of phage components and maturation and release of progeny phage particles from the cell.
- Phages play an important role in the transmission of genetic information between bacteria by the process of transduction. They can carry antibiotic resistance genes or virulence genes. Bacteria can be typed by phage typing methods for epidemiological purposes.

Questions

Essay

1. Describe the lytic cycle of bacteriophages.

Short Answers

1. Structure of a bacteriophage (draw and describe)
2. Phage typing (definition)
3. Lysogeny (definition)
4. Uses of bacteriophages

Part IV: Virology

Chapter 52

Hepatitis Viruses

Chapter navigation

- Hepatitis A virus (HAV)
- Hepatitis B virus (HBV)
- Hepatitis C virus (HCV)
- Hepatitis D virus (HDV) (delta virus)
- Hepatitis E virus (HEV)
- Hepatitis G virus (HGV)

INTRODUCTION

Many viruses cause hepatitis. The term 'viral hepatitis' refers to a primary infection of the liver by any one of a heterogeneous group of 'hepatitis viruses' which currently consist of types A, B, C, D, E and G.

Hepatitis viruses are taxonomically unrelated. Except for type B, which is a DNA virus, all the others are RNA viruses. The features common to them are their hepatotropism and ability to cause a similar icteric illness, ranging in severity from the unapparent to the fulminant fatal forms.

As all types of hepatitis viruses cause a clinically indistinguishable acute illness, their differentiation is based on their serological and molecular markers. Hepatitis may occur incidentally during many other viral infections, such as yellow fever, Lassa fever, Marburg, EB, cytomegalovirus, herpes simplex, varicella-zoster, measles, rubella or coxsackie viruses. These are not included in the category of viral hepatitis.

TYPES OF VIRAL HEPATITIS

Based on epidemiological and clinical criteria, the types of viral hepatitis are as follows:

Hepatitis A This occurs sporadically or as epidemics, affecting mainly children and young adults, and is transmitted through fecal contamination of water.

Hepatitis B A second type of viral hepatitis, transmitted mainly by

inoculation, was originally observed in persons receiving serum inoculation or blood transfusion.

The term **non-A non-B (NANB) hepatitis** was applied to infections that are now known to be caused by HCV, HDV and HEV. It is no longer used as it is possible to diagnose specific infections. These non-A non-B viruses include the following types:

- **Hepatitis C** was later identified as the cause of many non-A non-B transfusion-associated hepatitis cases.
- **Hepatitis D or delta virus** is a defective virus which depends on the helper functions of type B virus.
- **Hepatitis E** is yet another type of non-A non-B hepatitis transmitted by the fecal–oral route, prevalent mostly in the developing nations.
- **Hepatitis G** virus (HGV) is a rare cause of hepatic inflammation. It is a transfusion-transmitted agent but does not cause hepatitis. Although chronic infection and viremia have been documented, histologic evidence is rare, and serum aminotransferase levels are usually normal.

HEPATITIS A VIRUS (HAV)

Type A hepatitis (infectious hepatitis) is a subacute disease of global distribution, affecting mainly children and young adults.

In 1973, Feinstone and his co-workers, using immunoelectron microscopy (IEM), demonstrated this virus in the feces of experimentally infected human volunteers. Chimpanzees and marmosets can be infected experimentally. HAV can be grown in some human and simian cell cultures and is the only human hepatitis virus that can be cultivated in vitro. It has also been cloned.

Morphology

HAV is a 27–32 nm, non-enveloped RNA virus belonging to the picornavirus family. It is also designated as ‘enterovirus 72’ (Fig. 52.1). Because of its unique features, HAV is now recognised as the prototype of a new genus *Hepatovirus*. Only one serotype of the virus is known.

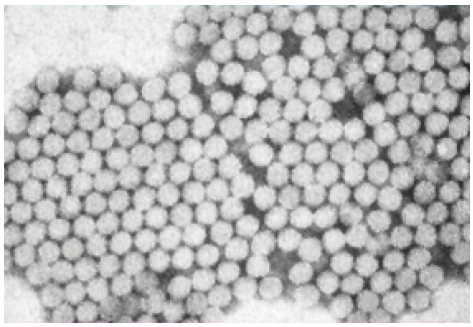


Fig. 52.1 Electron micrograph of 27 nm hepatitis A virus aggregated with antibody

Pathogenesis

HAV transmission is by the fecal–oral route. Infection is by ingestion. The virus multiplies in the intestinal epithelium and reaches the liver by hematogenous spread. It is shed in the feces during the late incubation period and prodromal phase of the illness. Once jaundice develops, it is rarely detectable in the feces. Chronic carriers are not seen. Virus persistence in nature depends on continuing inapparent infections.

A brief viremia occurs during the pre-icteric phase, but ceases with the onset of jaundice. Chronic viremia does not occur. Parenteral transmission is, therefore, very rare. Infection has been reported in recipients of some clotting factor concentrates. Transplacental infection has not been documented. HAV may be present occasionally in the saliva and urine of patients but this is not considered relevant in its spread.

Clinical Features

The large majority of infections are asymptomatic. Overt illness is seen in only about 5 per cent of those infected. The incubation period is 15–45 days. The clinical disease consists of two stages: the prodromal or pre-icteric and the icteric stages. Onset may be acute or insidious, with fever (100–102°F), malaise, anorexia, nausea and vomiting. Dark urine and clay-coloured stool may be seen by the patient in 1–5 days. These symptoms usually subside with the onset of jaundice. Yellow discolouration of the sclera and skin is seen when the serum bilirubin level is > 43 ∝mol/L. Rising levels may be associated with more severe disease. Enlarged liver with tenderness in the right upper quadrant with pain may be seen. Recovery is slow, over a period of 4–6 weeks. Very rarely, a **rapidly fatal fulminant hepatitis may occur**; most of the deaths occur in adults. The

disease is milder in children, in whom many infections may be anicteric. Mortality is low (0.1–1 per cent).

Resistance

HAV is inactivated by:
i) **heat** at 60°C for one hour,
ii) **ether and acid** at pH 3,
iii) **boiling** for one minute,
iv) **1:4,000 formaldehyde** at 37°C for 72 hours and
v) Chlorine (bleach) 1 ppm for 30 minutes.

It is not affected by anionic detergents and survives prolonged storage at a temperature of 4°C or below.

Epidemiology

Natural infection with HAV is seen only in humans. Though primates such as chimpanzees have been shown to acquire the infection from humans and transmit it to human contacts, there is no evidence of any extra human source of the virus in nature.

Hepatitis A occurs **sporadically** or as **outbreaks**, which may be caused as mentioned by contaminated food, water or milk. Shellfish have been known to be responsible for outbreaks. Domestic or institutional spread of infection among children is common. Person-to-person spread is increased by poor personal hygiene and overcrowding. The **epidemiology of hepatitis A resembles that of poliomyelitis**. In the developing countries, infection is acquired in childhood and by the age of 10, 90 per cent of the population possess the antibody to the virus and are immune. **In India, type A hepatitis is the most common cause of acute hepatitis in children** but is much less frequent in adults. In the temperate regions, the disease shows an autumn–winter predilection, whereas in the tropics, no seasonal distribution is evident. In India, the disease tends to be associated with heavy rainfall.

Laboratory Diagnosis

The mainstay of diagnosis is detecting altered liver enzymes, increased bilirubin and specific IgM antibodies in the early phase of the infection.

1. **Specimen** Blood, serum or feces may be collected for demonstration of altered liver function tests, antibody or the virus respectively.

2. **Liver function tests** Serum aminotransferase and aspartate aminotransferase are elevated. Aspartate transaminase (AST) and alanine aminotransferase (ALT) show variable increase initially prior to increase in serum bilirubin.

3. **Serology** Diagnosis is usually by the detection of antibody. IgM anti-HAV antibody appears during the late incubation period, reaches peak levels in 2–3 weeks and disappears after 3–4 months. The IgG antibody appears at about the same time, peaks in 3–4 months and persists much longer, perhaps for life.

Interpretation

- Demonstration of IgM antibody in serum indicates current or recent infection.
- IgG antibody denotes recent or remote infection and immunity.
- ELISA kits for the detection of IgM and IgG antibodies are available (Fig. 52.2).

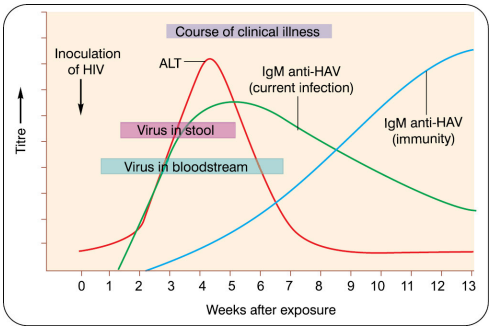


Fig. 52.2 Immunologic and biologic events associated with hepatitis A virus infection in humans

4. **Direct demonstration of the virus** The virus can be visualised by IEM in fecal extracts during the late incubation period and the pre-icteric phase but seldom later. This is not commonly used for diagnosis.

5. **Culture** HA virus can be cultivated on human cell lines and fetal monkey cell lines (FRhK-4/R) but is seldom used for laboratory diagnosis.

6. **PCR** Viral RNA can be detected from stool filtrates by reverse transcriptase PCR (RT-PCR) or real-time PCR. However, this method of

diagnosis is not cost-effective as most often, the infection is self-limiting.

Prophylaxis

General prophylaxis consists of improved sanitary practices and prevention of fecal contamination of food and water. A safe and effective formalin-inactivated, alum-conjugated vaccine containing HAV grown in human diploid cell culture is available. A full course consists of two intramuscular injections of the vaccine, followed by a booster dose after 10–12 months. This is commonly administered to children between 2 and 18 years.

Adults travelling to endemic areas can also be vaccinated. Protection begins 4 weeks after injection and lasts for 10–20 years.

A combination of HAV and HBV vaccine, Twinrix, with the same immunogens as the individual vaccines and which immunises against both is available.

Specific passive prophylaxis Immunisation with IgG-containing anti-HAV, at concentrations sufficient to protect (16% solution in a dose of 0.2–0.12 mL/kg body weight IM) before exposure or in the early incubation period, can prevent or attenuate clinical illness while not necessarily preventing infection and virus excretion.

Natural infection with HAV, clinical or subclinical, leads to lifelong immunity. There is no cross-immunity between HAV and any of the other hepatitis viruses.

Treatment

This is symptomatic. No specific antiviral drug is available. Liver transplantation may be an option in selected cases if the patient has fulminant hepatic failure.

HEPATITIS B VIRUS

The discovery of HBV was serendipitous. In 1965, Blumberg, while studying human serum lipoprotein allotypes, observed in the serum of an Australian aborigine, a new antigen which gave a clearly defined line of precipitation with sera from two hemophiliacs who had received multiple blood transfusions. This was named the **Australia antigen**. By 1968, it was

found to be associated with serum hepatitis. It was subsequently shown to be the surface component of HBV. Therefore, the name Australia antigen was changed to **hepatitis B surface antigen (HBsAg)**.

Hepatitis B is the most widespread and the most important type of viral hepatitis. More than a third of the world's population is estimated to have been infected by HBV. About a quarter of them become HBV carriers. A quarter of these develop serious liver disease, including chronic hepatitis, cirrhosis and primary hepatic cancer. As there is an effective vaccine against HBV, hepatocellular carcinoma has become the only human cancer that is vaccine-preventable. The WHO estimates that HBV infection causes more than a million deaths a year worldwide.

Clinical Case

A 40-year-old woman who had received multiple blood transfusions over the previous six months presented with persistent fatigue, loss of appetite, nausea, vomiting and abdominal pain for a duration of 10 days. She had a history of passing high-coloured urine. Her liver function tests showed elevated serum bilirubin and liver enzymes. A viral hepatitis panel was advised. The tests showed a positive HBsAg, positive for anti-HBc-IgM. It was negative for anti-HAV-IgM and anti-HCV-IgM. She was diagnosed with acute HBV infection, and was placed on supportive therapy.Her liver enzymes and viral markers were monitored every month to check for seroconversion from HBsAg to anti-HBsAg positivity (indicative of resolution of the infection).

Morphology

HBV is a 42 nm DNA virus with an outer envelope and an inner core, 27 nm in diameter, enclosing the viral genome and a DNA polymerase (Fig. 52.3). Because of its unique features, HBV is assigned to a separate family, Hepadnaviridae (hepatotropic DNA viruses), which consists of two genera: *Orthohepadnavirus,* containing HBV as well as the woodchuck and ground squirrel hepatitis viruses, and *Avihepadnavirus*, containing the Pekin duck and grey heron hepatitis viruses. HBV is hepadnavirus type 1.

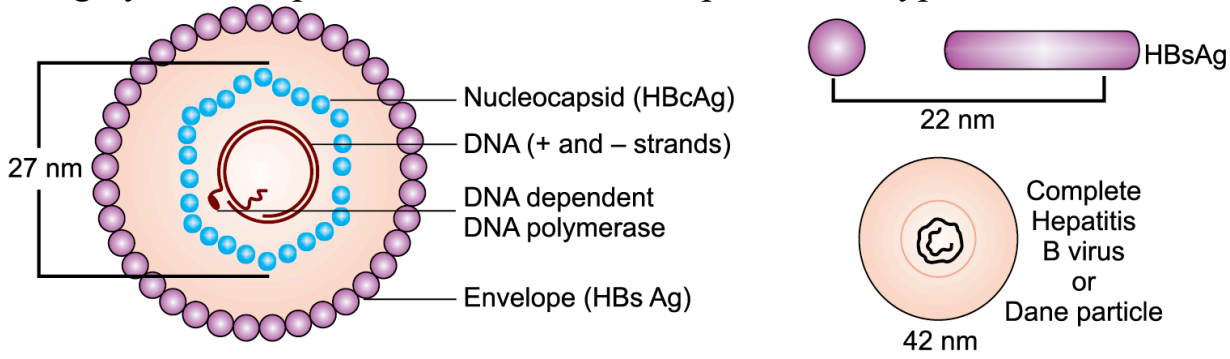


Fig. 52.3 Structure of hepatitis B virus

Under the electron microscope, sera from type B hepatitis patients show three types of particles (Fig. 52.4):

- **Spherical particle, the first type,** is the most abundant form. It is 22 nm in diameter.
- The second type of particle is **filamentous or tubular** with a diameter of 22 nm and varying length. These two particles are antigenically identical and are surface components of HBV (HBsAg) that are produced in great excess.
- The third type of particle, far fewer in number, is a double-walled spherical structure, 42 nm in diameter. This particle is the complete hepatitis B virus. **It was first described by Dane in 1970 and so is known as the Dane particle** (Fig. 52.4a and b).

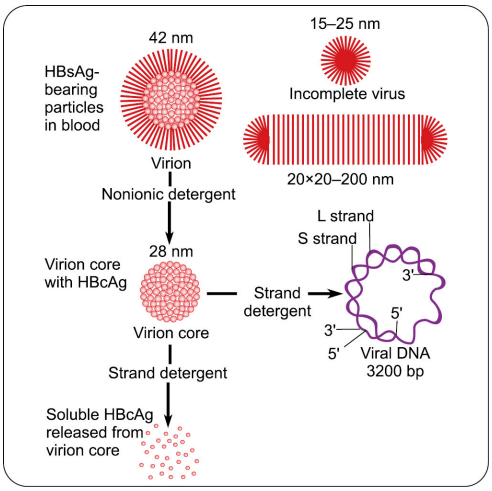


Fig. 52.5 Structural components of HBV

The envelope proteins expressed on the surface of the virion and the surplus 22-nm-diameter spherical and filamentous particles constitute the hepatitis B surface antigen. HBsAg consists of two major polypeptides, one of which is glycosylated.

Antigenic Diversity

HBsAg exhibits antigenic diversity. It contains two different antigenic components: the common group-reactive antigen a, and two pairs of type-specific antigens d-y and w-r, only one member of each pair being present

at a time. HBsAg can thus be divided into four major antigenic subtypes:

1. **adw**
2. **adr**
3. **ayw**
4. **ayr**

The subtypes breed true, and the index case and contacts in an outbreak have the same subtype. They show a distinct geographical distribution.

Subtype ayw is common from West Asia through the Middle East to western and northern India; **subtype adw** is common in Europe, Australia and the Americas; **adr** is prevalent in South and East India and the Far East; **ayr** is very rare.

Surface antigenic reactivities (a, x, f, t, j, n, g) have been reported but they have not been adequately studied.

HBcAg Mild detergent treatment disrupts the viral envelope and exposes the core or nucleocapsid. The antigen expressed on the core is called the hepatitis B core antigen (HBcAg) (Fig. 52.5).

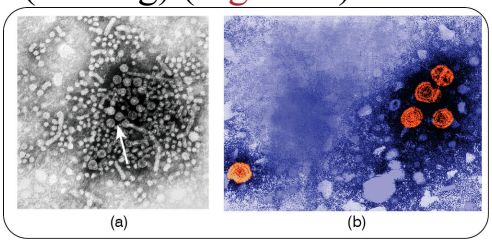


Fig. 52.4 (a) Hepatitis B virus and (b) Dane particles
(*Source:* (a) Public Health Image Library, ID 10229 / Betty Partin / CDC and (b) Public Health Image Library, ID 10755 / Dr. Erskine Palmer / CDC)

HBeAg A third antigen called the hepatitis B antigen (HBeAg) is a soluble, non-particulate nucleocapsid protein. HBcAg and HBeAg, though immunologically distinct, are coded for by the same gene.

Viral Genome

The nucleocapsid encloses the viral genome consisting of two linear strands of DNA held in a circular configuration. One of the strands (the plus strand) is incomplete and so the DNA appears partially double-stranded and partially single-stranded. Associated with the **plus strand** is a viral **DNA polymerase**, which has both DNA-dependent DNA polymerase and RNA-dependent reverse transcriptase functions. This polymerase can repair the

gap in the plus strand and render the genome fully double-stranded. The genome has a compact structure with four overlapping genes (Fig. 52.6).

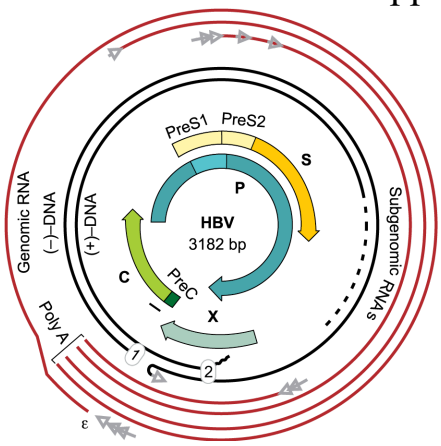


Fig. 52.6 Genetic organisation of the HBV genome

The ***S* gene** codes for the surface antigen. It consists of the S region and two Pre-S regions: Pre-S2 and Pre-S1. The protein coded for by the S region is called the S or major protein. When translation begins from the Pre-S2 region, the M or middle protein is formed. When the entire gene from Pre-S1 is translated, the L or large protein results. The L protein is present only in the virion, while the M and S proteins are found in the circulating HBsAg particles also.

The ***C* gene** has two regions, C and Pre-C. When the C region alone is translated, the core antigen (HBcAg) is formed. HBcAg is assembled as the nucleocapsid core particles. It is not secreted and does not circulate in blood, but can be demonstrated in hepatocytes by immunofluorescence. Antibodies to HBc, both IgM and IgG, appear in blood. The IgG antibody to HBcAg persists in blood long after all other serological markers have disappeared and so provides a useful marker of prior infection with HBV. If translation begins from the Pre-C region, the resulting protein is HBeAg, a non-particulate soluble antigen possessing a signal protein which enables it to be secreted. It is, therefore, present in circulation. The presence of HBeAg in blood provides a convenient and readily detectable marker of HBV replication and high infectivity.

The ***P* gene** is the largest and codes for the DNA polymerase enzyme.

The ***X* gene** codes for a small non-particulate protein (HBxAg), which has transactivating effects on both viral and some cellular genes. This leads to enhanced replication of HBV, as well as of some other viruses, such as the

human immunodeficiency virus. HBxAg and its antibody are present in patients with severe chronic hepatitis and hepatocellular carcinoma.

Mutations

A few cases of infection by mutant viruses have been identified. Two types of mutations have been studied:

Pre-core mutants One type, initially identified in Mediterranean countries, presents as severe chronic hepatitis, caused by pre-core mutants unable to synthesise HBeAg. Those infected with **pre-core mutants** may be positive for anti-HBe and anti-HBc.

Escape mutants The second group have been seen in some infants born to HBeAg-positive mothers, and in liver transplant recipients who had received combined immunisation with anti-HBV immunoglobulin and vaccine. They show mutation in the common antigenic determinant of HBsAg, preventing them from being neutralised by the anti-HBsAg antibody. If such mutants become more common, they may pose problems in hepatitis B prophylaxis.

YMDD mutant A third type of mutant has emerged following Lamivudine treatment. Most Lamivudine-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase.

Pathogenesis

The pathogenesis of hepatitis appears to be immune-mediated. Hepatocytes carry viral antigens and are subject to antibody-dependent NK cell and cytotoxic T cell attack. In the absence of adequate immune response, HBV infection may not cause hepatitis but may lead to carrier state. Therefore, infants and immunodeficient persons are more likely to become asymptomatic carriers following infection.

Clinical Features

The incubation period is long, about 1–6 months. The clinical picture of hepatitis B is similar to that of type A, but it tends to be more severe and protracted. Onset is insidious and fever is not prominent. Extrahepatic complications like arthralgia, urticaria and rarely, polyarteritis or

glomerulonephritis may occur. These are ascribed to circulating immune complexes containing the viral surface antigen.

Outcome After Hepatitis B Infection

About 90–95 per cent of adults with acute hepatitis B infection recover within 1–2 months of onset and eliminate the virus from the body within about six months, remaining immune thereafter. Mortality is about 0.5–2.0 per cent, but may be more in post-transfusion cases. About 1 per cent of patients, particularly those having **simultaneous delta virus infection, develop fatal fulminant hepatitis**. They may develop hepatic encephalopathy within a short period of infection.

A proportion of cases (1–10 per cent) remain chronically infected. They may be asymptomatic carriers or may progress to recurrent or chronic liver disease or cirrhosis. Immune tolerance or immunological clearance are the outcomes. A few of them may get reactivated or develop hepatocellular carcinoma after many decades (Fig. 52.7).

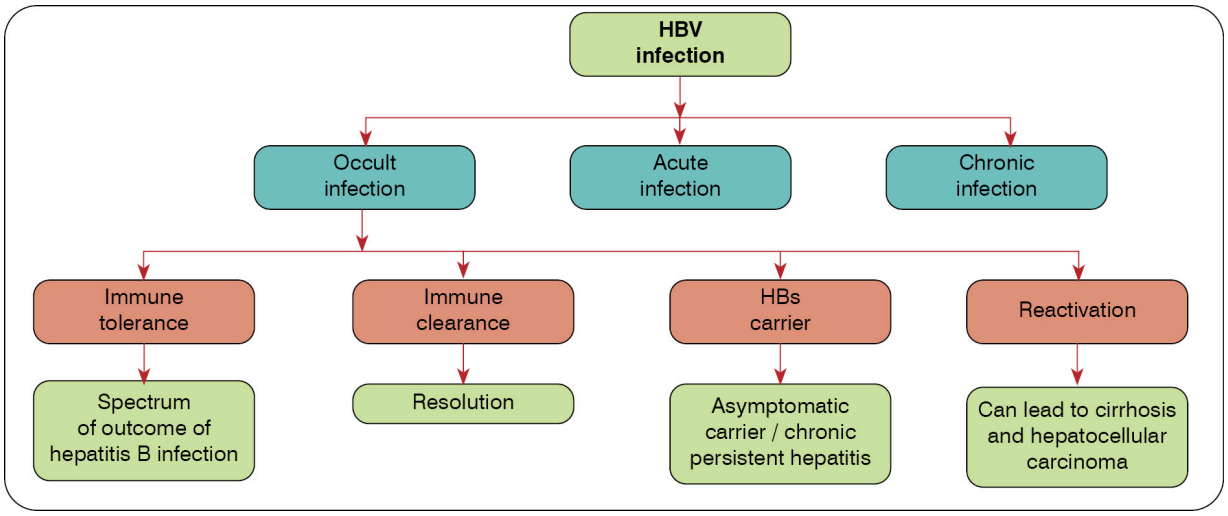


Fig. 52.7 Flowchart of possible outcomes in hepatitis B infection

Replication

HBV replicates within hepatocytes. Viral DNA exists in the hepatocyte nucleus in the free extrachromosomal state or integrated with the cell chromosome. Replication resembles that seen in retroviruses, in that DNA is synthesised from an RNA template by reverse transcription.

HBV DNA and protein have also been identified in extrahepatic sites such

as the bone marrow, spleen, lymph nodes and circulating lymphocytes but no apparent damage is produced in these locations. The significance of this extrahepatic presence is not understood.

HBV does not grow in any conventional culture system. However, limited production of the virus and its proteins can be obtained from several cell lines transfected with HBV DNA. **HBV proteins have been cloned in bacteria and yeast**. The chimpanzee is susceptible to experimental infection and can be used as a laboratory model.

Resistance

HBV is a relatively heat-stable virus. It remains viable at room temperature for long periods. Heat at 60°C for 10 hours reduces infectivity by 100- to 1,000-fold. It is susceptible to chemical agents. Exposure to hypochlorite (10,000 ppm available chlorine) or 2% glutaraldehyde inactivates infectivity, though HBsAg may not be destroyed by such treatment.

Epidemiology

Hepatitis B exists across the world. Natural infection occurs only in humans. There is no animal reservoir. The virus is maintained in the large pool of carriers whose blood contains circulating viruses for long periods, in some, even lifelong. The infection is usually sporadic, though occasional outbreaks have occurred in hospitals, orphanages and institutions for the mentally handicapped.

The prevalence of hepatitis carriers varies widely in different countries, in relation to their living standards. India falls in the intermediate group: carrier rate
2–7 per cent, with higher carrier rates in the southern part of the country and lower rates in the northern part.

Carrier

A **hepatitis carrier** is a person with detectable HBsAg in his blood for more than six months. Following infection, about 5–10 per cent of adults, 30 per cent of children and 90 per cent of neonates become carriers. The carrier state is more common among males. There are over 350 million carriers now worldwide. Of them, about 45 million are in India.

Carriers are of two categories:

i) **Super carriers** are highly infectious, having high-titre HBsAg, along with HBeAg, DNA polymerase and HBV in circulation, and generally elevated transaminases. Some of them have enormous antigenemia and viremia, up to 10^{13} HBsAg particles equal to 500 mg of protein, and 10^{8} HBV per mL of blood. About a quarter of the carriers in India are HBeAg-positive.

ii) **Simple carriers** have low infectivity and low-titre HBsAg in blood, with negative HBeAg, HBV and DNA polymerase. Many super carriers, in time, become simple carriers.

Transmission

HBV is a bloodborne virus and the infection is transmitted by parenteral, sexual and perinatal modes.

Parenteral transfusion The blood of carriers, and less often, that of patients, is the most important source of infection. The virus may also be present in other body fluids and excretions, such as saliva, breast milk, semen, vaginal secretions, urine, bile and feces. Of these, semen and saliva are known to transmit the infection. Feces is not known to be infectious.

Transfusion of carrier blood, once the most widely known mode of infection has largely been eliminated wherever donor screening is strictly enforced. However, HBsAg screening is not a totally fail-safe method as infection has occurred even with HBsAg-negative, anti-HBc-positive blood, which may have had undetectable amounts of the virus.

HBV is very highly infectious, far more than HIV. Any object or procedure than can convey minute traces of infected blood or other material, as little as 0.00001 mL, can be infectious.

Coinfection with hepatitis B virus (HBV) and HIV is common, with 70–90% of HIV-infected individuals in the United States having evidence of past or active infection with HBV. Both the infections are endemic in India. However, there is no countrywide data on the coinfection of HBV and HIV. Factors affecting the prevalence of chronic HBV include age at the time of infection and mode of acquisition, which vary geographically.

The course of acute HBV may be modified in the presence of HIV infection, with a lower incidence of icteric illness and lower rates of spontaneous clearance of HBV. Persons with HIV and chronic HBV coinfection have higher levels of HBV DNA and lower rates of clearance of

the hepatitis Be antigen.

Modes of transmission can also be through shared syringes, needles and other sharp items or endoscopes, personal articles such as razors, nail clippers or combs, and practices such as acupuncture, tattooing, ritual circumcision, ear or nose piercing, and field camps for surgery or disease detection by blood testing where separate sterile articles may not be available. Professionals using sharp articles like barbers, dentists and doctors may unwittingly transmit the virus if great care is not taken.

Infection by direct contact with open skin lesions such as pyoderma, eczema, cuts and scratches is very common among young children in developing countries, as also through household transmission where opportunities exist for contact with blood or saliva among members.

Perinatal transmission Congenital or vertical transmission is quite common from carrier mothers. The risk to babies is high if the mother is HBeAg-positive (60–90 per cent) and low if negative (5–15 per cent). True congenital infection (in utero, transplacental) is rare. Infection is usually acquired during birth by contact of maternal blood with the skin and mucosa of the fetus, or in the immediate postnatal period. Infection by ingestion has been reported, but its efficacy is very low. However, it is safer if carrier mothers do not breastfeed when proper nutrition of their babies can be otherwise ensured. **HBV-infected neonates generally do not suffer from any clinical illness** but remain carriers for life and some of them may develop hepatocellular carcinoma after many decades.

Sexual transmission The risk of transmission by heterosexual and homosexual contact increases with the number of partners and the duration of such relationships. HBV infection has occurred after artificial insemination. Semen donor screening is, therefore, obligatory.

Occupational risk Certain groups and occupations carry a high risk of infection. These include medical and paramedical personnel, staff of blood banks, dialysis units, medical laboratories and mental health institutions, barbers and sex workers. Dentists and doctors have been responsible for small outbreaks.

Laboratory Diagnosis

Detection of viral markers Specific diagnosis of hepatitis B rests on the

serological demonstration of the viral markers. It is, therefore, necessary to understand the sequence of their appearance in blood (Fig. 52.8).

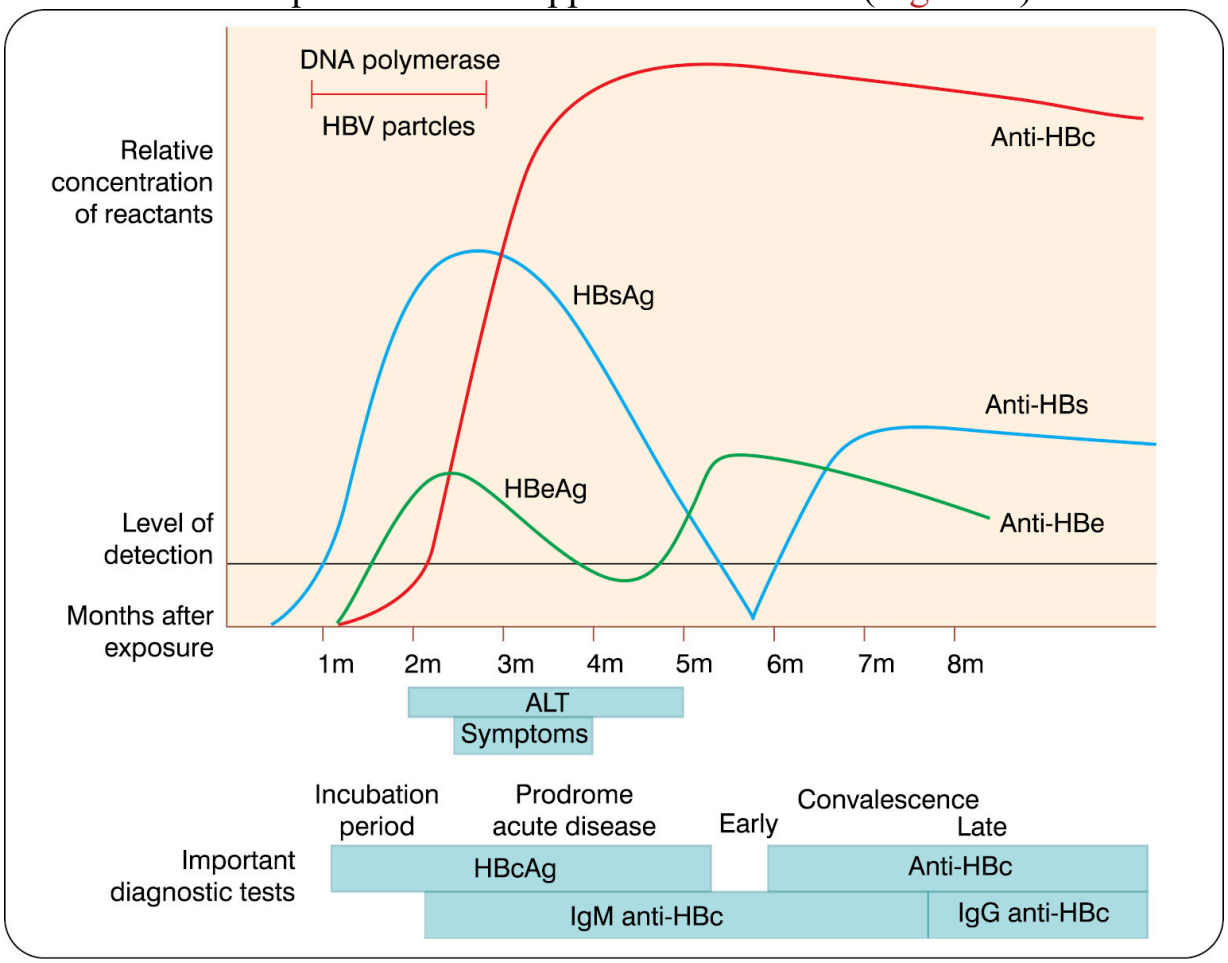


Fig. 52.8 Clinical and serologic events occurring in a patient with acute hepatitis B virus infection

HBsAg is the **first marker to appear in blood after infection**, being detectable even before the elevation of transaminases and onset of clinical illness. It remains in circulation throughout the icteric or symptomatic course of the disease. In the typical case, it disappears within about two months of the start of clinical disease, but may sometimes last for six months and even beyond. When it is no longer detectable, the antibody, anti-HBs, appears and remains for very long periods. **Anti-HBs is the protective antibody**.

HBcAg is **not demonstrable in circulation because it is enclosed within** the HBsAg coat, but its antibody, anti-HBc, appears in serum a week or two after the appearance of HBsAg. It is, therefore, **the earliest antibody marker to be seen in blood**, long before anti-HBe or anti-HBs. As anti-

HBc remains lifelong, it serves as a useful indicator of prior infection with HBV, even after all the other viral markers become undetectable. Initially, anti-HBc is predominantly IgM, but after about six months, it is mainly IgG. Selective tests for IgM or IgG anti-HBc, therefore, enable distinction between recent or remote infection respectively.

HBeAg appears in blood concurrently with HBsAg, or soon afterwards. Circulating **HBeAg is an indicator of active intrahepatic viral replication**.

DNA polymerase, HBV DNA and virions in blood reflects high infectivity. The disappearance of HBeAg coincides with a fall of transaminase levels in blood. It is followed by the appearance of anti-HBe.

For the diagnosis of HBV infection, detection of HBsAg in blood is all that is ordinarily necessary. The simultaneous presence of IgM anti-HBc indicates recent infection and the presence of IgG anti-HBc, remote infection. Occasionally, when the level of HBsAg is too low to be detectable, diagnosis has to be made by testing for IgM anti-HBc (*Clinical case*). Sometimes, due to mutations in the surface antigen gene, HBsAg becomes undetectable. However, the anti-hepatitis core (anti-HBc) antibody can be detected in the serum. This phenomenon is called **occult hepatitis B infection** (OBI).

HBeAg provides information about relative infectivity. Its presence denotes high infectivity and its absence, along with the presence of anti-HBe, indicates low infectivity. As it is invariably present during acute hepatitis, its testing is indicated only in chronic infection and carriers.

The presence of anti-HBs without any other serological virus marker indicates immunity following vaccination. Table 52.1 shows the interpretation of various serological patterns in hepatitis B.

Like HBeAg, **HBV DNA is also an indicator of viral replication and infectivity**. Molecular methods such as DNA:DNA hybridisation and PCR, at present used for HBV DNA testing, are highly sensitive and quantitative. HBV DNA level in serum reflects the degree of viral replication in the liver and so helps to assess the progress of patients with chronic hepatitis under antiviral chemotherapy.

Table 52.1 Interpretation of common serological patterns in HBV infection

Virus/antibody markers	Interpretation

HBsAg	HBeAg	Anti-HBc	Anti-HBs	Anti-Hbe	
+	+	IgM	–	–	Acute HBV infection; highly infectious
+	+	IgG	–	–	Late/chronic HBV infection or carrier state; highly infectious
+	–	IgG	–	+/–	Late/chronic HBV infection or carrier state; low infectivity
–	+/–	IgM	–	+/–	Seen rarely in early acute HBV infection; infectious
–	–	IgG	+/–	+/–	Remote HBV infection; infectivity nil or very low
–	–	–	+	–	Immunity following HBV vaccine

Prophylaxis

General prophylaxis consists of avoiding risky practices like promiscuous sex, injectable drug abuse and direct or indirect contact with blood, semen or other body fluids of patients and carriers. Health education, the use of disposable syringes and needles, screening of blood, semen and organ donors, have all helped to an extent, but these alone cannot eliminate the risk altogether, particularly in the developing countries.

Immunisation

The only certain method appears to be universal immunisation. Both passive and active methods of immunisation are available.

Passive immunisation by hyperimmune hepatitis B immune globulin (HBIG) This is prepared from the plasma of human volunteers with high-titre anti-HBs, administered IM in a dose of 300–500 I.U. soon after exposure to infection. It may not prevent infection, but protects against illness and the carrier state.

Active immunisation by Hepatitis B vaccine (HBV)

The vaccine currently preferred is **genetically engineered** by cloning the *S* gene of HBV in baker's yeast. It consists of non-glycosylated HbsAg particles alone. It is given with alum adjuvant, IM into the deltoid or, in infants, into the anterolateral aspect of the thigh. Gluteal injection is not recommended as it may result in poor immune response.

Three doses given at 0, 1 and 6 months constitute the full course.

Seroconversion occurs in about 90 per cent of the vaccinees.

A vaccine containing all antigenic components of HBsAg (Pre-S1, Pre-S2 and S) has been developed, which gives greater seroconversion. Seroconversion can be checked by testing for anti-HBs, which is usually detectable for about five years. Clinical protection is believed to last much longer. Booster doses are needed only for those at high risk.

Now that the vaccine is manufactured in India, and is available at lower cost, it has been included in **the national immunisation schedule.** 0.5 mL is given in the anterolateral side of the mid-thigh of the baby at birth or within 24 hours.

Combined immunisation For non-immune persons exposed to HBV, combined immunisation is recommended. For babies born to carrier mothers, a single injection of 0.5 mL of HBIG given IM immediately after birth is followed by the full course of vaccine at a different anatomical site, the first dose being given within 12 hours of birth. When HBIG is not available, the vaccine given alone has been reported to provide protection.

For individuals undergoing hemodialysis, annual testing for anti-HBs is recommended.

PEP guidelines

- For unvaccinated individuals exposed to the virus through needle stick injury or exposure to blood and body fluids of infected persons, a combination of HBIG and hepatitis B vaccine is advised. A single dose of 0.06 mL/kg is given immediately after exposure, followed by the full course of hepatitis B vaccination.
- In immunocompromised individuals, after the regimen mentioned above, a booster dose is recommended.
- Individuals with undetectable antibodies (< 1:10) are also advised to undergo repeat vaccination.

Treatment

No specific antiviral treatment is available for acute HBV infection. Entecavir, tenofovir disoproxil fumarate (TDF), and peginterferon (peg-IFN), alone or in combination with other antiviral agents such as lamivudine and famciclovir, has been beneficial in some cases of chronic hepatitis. Tenofovir alafenamide (TAF) is recommended for CHB (chronic hepatitis B) in adults. There is no effective treatment for the carrier state, though spontaneous resolution takes place in some of them.

HEPATITIS C VIRUS (HCV)

Attempts to identify the group of 'non-A non-B' viruses by experimental infection in chimpanzees led to the discovery of hepatitis C virus (HCV). It is now the most common cause of post-transfusion hepatitis in the developed countries.

Morphology

The virus has not been grown in culture but has been cloned in *Escherichia coli*. HCV is a 50–60 nm virus with a linear, single-stranded RNA genome, enclosed within a core and surrounded by an envelope, carrying glycoprotein spikes (Fig. 52.9). HCV resembles flaviviruses in structure and organisation and has been classified as a new genus *Hepacivirus* in the family Flaviviridae.

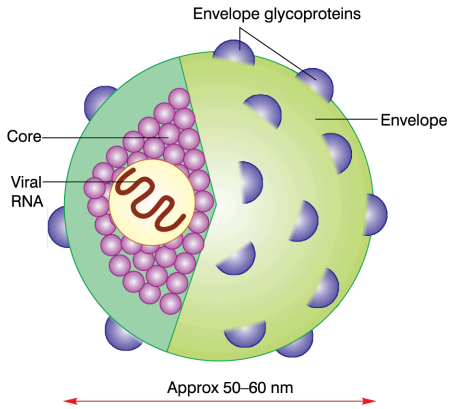


Fig. 52.9 HCV virus with its core viral RNA, envelope and glycoprotein

The virus shows considerable genetic and antigenic diversity. At least **six different genotypes** and many subtypes have been identified, indicating high mutability. Some genotypes are seen worldwide, while others are localised. Because of this diversity, there is little heterologous or even homologous post-infection immunity in hepatitis C.

Clinical Features

The incubation period is long, 15–160 days, with a mean of 50 days. The **acute illness** is usually mild or anicteric. Overt jaundice is seen in about 5 per cent of patients only. The important part in type C hepatitis is the **chronic illness**. About 50–80 per cent of patients progress to chronic hepatitis, with some developing **cirrhosis and hepatocellular carcinoma.**

Epidemiology

HCV infection is seen only in humans. The source of infection is the large number of carriers, estimated to be about 200 million worldwide. In general, the epidemiology resembles that of hepatitis B.

The transmission of this infection is mainly by blood transfusion and other modes of contact with infected blood or blood products. Injectable drug abusers, transplant recipients and immunocompromised persons are at high risk. Sexual transmission is probably less important. Vertical transmission from mother to baby may take place.

The infection occurs throughout the world, with carrier rates of 1–20 per cent. HCV infection is prevalent in India too, with an estimated 12.5 million cases. A quarter of all chronic hepatitis cases in India are believed to be due to HCV infection.

Laboratory Diagnosis

1. **Serum antibody detection** The standard method of diagnosis is antibody detection by ELISA. The antigens used are various structural and non-structural proteins cloned in *E.coli.* Three successive generations of such antigens have been introduced to improve sensitivity and specificity of serological diagnosis. Even the **third-generation ELISA** currently in use, employing NS-5 region protein and synthetic peptides, becomes positive only months after the infection and shows non-specific reactions. Confirmation by immunoblot assay is therefore recommended. In HCV infection, antibodies appear irregularly and late, limiting their diagnostic utility
(Fig. 52.10). Culture is not yet established.

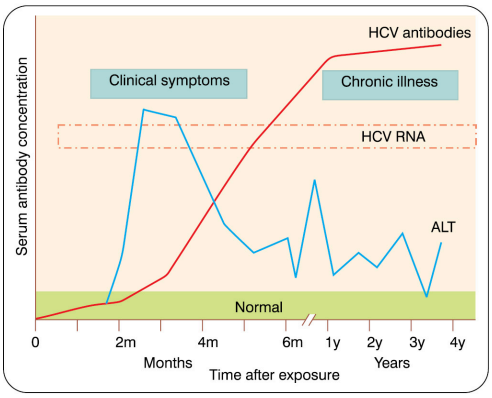


Fig. 52.10 Course of HCV infection and appearance of HCV RNA and antibodies

2. **Identification of HCV RNA in blood** This provides more sensitive and specific results within a few days of exposure to HCV. Molecular methods like PCR and branched DNA assay are employed for the purpose.

Prophylaxis

Only general prophylaxis, such as blood screening, is possible. No specific active or passive immunising agent is available.

Treatment

The choice of medications and length of treatment depend on the hepatitis C genotype, presence of existing liver damage, other medical conditions and prior treatments. Recombinant IFN-α either alone or in combination with antiviral agents like ribavirin has been reported to be useful in some cases. Two new drugs for hepatitis C infection have been approved in the USA. They are protease inhibitors Boceprevir and Telaprevir. Sofosbuvir–ledipasvir is a combination medicine used to treat hepatitis C.

HEPATITIS D (DELTA) VIRUS (HDV)

Hepatitis D virus (HDV), also known as delta agent, is a defective RNA virus dependent on the helper function of HBV for its replication and expression.

Morphology

HDV is a spherical, 36-nm particle with an outer coat composed of the hepatitis B surface antigen surrounding the circular single-stranded RNA genome. Though it resembles some plant viruses, such as viroids or satellite viruses, it is classified in a new genus, *Deltavirus*, because of its special features.

Clinical Features

Its mode of transmission is the same as for HBV. Two types of infection are recognised:

Co-infection Here, delta and HBV are transmitted together at the same time. Co-infection clinically presents as acute hepatitis B, ranging from mild to fulminant disease.

Superinfection Here, delta infection occurs in a person already harbouring HBV. It usually leads to more serious and chronic illness, with deterioration of the underlying HBV infection. No association has been noted between HDV and hepatocellular carcinoma.

Laboratory Diagnosis

The delta antigen is primarily expressed in liver cell nuclei, where it can be demonstrated by immunofluorescence. It is only occasionally present in serum. **Anti-delta antibodies** appear in the serum and can be identified by ELISA. The IgM antibody appears 2–3 weeks after infection and is soon replaced by the IgG antibody in acute delta infection. However, in chronic infection, the IgM antibody persists for years. Delta RNA sequences have been cloned and DNA probes have been developed for the rapid identification of delta particles in circulation.

Epidemiology

HDV is distributed worldwide but is more common in certain endemic areas. In the Mediterranean countries where it is **endemic**, infection is spread commonly by non-percutaneous routes, especially by close personal contact. In the **non-endemic** areas, such as northern Europe and North America, infection is more often through blood and blood products and is commonly seen in drug addicts and hemophiliacs. Introduction of HDV into non-endemic areas where HBV infection is common may lead to outbreaks of severe hepatitis with high mortality.

Prophylaxis

No specific prophylaxis exists, **but immunisation with the HBV vaccine is effective as HDV cannot infect persons immune to HBV**. Screening of blood donors for HBsAg automatically limits bloodborne HDV infection.

HEPATITIS E VIRUS (HEV)

The bulk of hepatitis in the developing countries is transmitted enterically through fecal pollution of drinking water (hence the name **enterically-transmitted NANB or E-NANB**). It often appears as epidemics (hence also called **epidemic NANB**). The largest such epidemic occurred in Delhi

during the winter of 1955–56, affecting over 30,000 persons within six weeks. Several outbreaks and sporadic infections have been reported from many parts of the Indian subcontinent, Central and South Asia, North Africa and Central America. The disease is now called **type E hepatitis** and its causative agent, hepatitis E virus (**HEV**). In India, HEV is responsible for the majority of epidemic and sporadic hepatitis in adults.

The source of infection is fecal contamination of drinking water and the environment. Secondary attack rate among household contacts is very low in type E hepatitis, 2–3 per cent as against 10–20 per cent in HAV infection.

Morphology

HEV is a spherical non-enveloped virus, 32–34 nm in diameter, with a single-stranded RNA genome. The surface of the virion shows indentation and spikes. The virus is very labile. In morphology and physical characteristics, it resembles Caliciviruses such as the Norwalk virus. It has been provisionally classified under *Hepeviridae*.

Clinical Features

The incubation period range is 2–9 weeks with an average of six weeks. Most cases occur in the young to middle-aged adults (15–40 years old). The disease is generally mild and self-limiting, with a low case fatality of about 1 per cent. A unique feature is the clinical severity and high case fatality rate of 20–40 per cent in pregnant women, especially in the last trimester of pregnancy.

Laboratory Diagnosis

HEV can be demonstrated by immune-electron microscopy (IEM) in the bile and feces of patients in the incubation period or acute phase of illness. The carrier state has not been observed. Only one serotype of the virus exists. ELISA kits are available for the detection of IgG and IgM antibodies, using recombinant and synthetic peptide antigens.

Table 52.2 lists the comparative features of the various viral hepatitis types.

Table 52.2 Viral hepatitis types: comparative features

	A	B	C	D	E
Virus	HAV, 27 nm RNA, Picornavirus (*Hepatovirus*)	HBV, 42 nm DNA (*Hepadnavirus*)	HCV, 50–60 nm RNA, *Flavivirus* (*Hepacivirus*)	HDV, 35–37 nm Defective RNA *Deltavirus*	HEV, 32–34 nm RNA *Hepevirus*
Modes of infection	Fecal–oral	Percutaneous, vertical, sexual	Percutaneous	Percutaneous	Fecal–oral
Affected age group	Children	Any age	Adults	Any age	Young adults
Incubation period (days)	15–45	30–180	15–160	30–180	15–60
Onset	Acute	Insidious	Insidious	Insidious	Acute
Illness	Mild	Occasionally severe	Moderate	Occasionally severe	Mild, except in pregnancy
Carrier state	Nil	Common	Present	Nil (only with HBV)	Nil
Oncogenicity	Nil	Present specially after neonatal infection	Present	Nil	Nil
Prevalence	Worldwide	Worldwide	Probably worldwide	Endemic areas (Mediterranean, N. Europe, Central and N. America)	Only developing countries (India, Asia, Africa, Central, America)
Specific prophylaxis	Ig and vaccine	Ig and vaccine	Nil	HBV vaccine	Nil

HEPATITIS G VIRUS (HGV)

Two flavivirus-like isolates were obtained in 1995 from Tamarin monkeys inoculated with blood from a young surgeon (GB) with acute hepatitis. A similar virus was isolated from another human specimen the same year. These isolates were called GB viruses A, B and C, respectively.

In 1996, an isolate closely resembling GBV-C was obtained from a patient with chronic hepatitis. This has been called the hepatitis G virus (HGV). It has not been grown but its RNA genome has been cloned. HGV RNA has been found in patients with acute, chronic and fulminant hepatitis, hemophiliacs, patients who had undergone multiple transfusions and hemodialysis, intravenous drug addicts and blood donors. HGV appears to be a bloodborne virus resembling HCV. Its prevalence is higher in patients infected with HIV and HCV. Its role in hepatitis is yet to be clarified.

HGV RNA can be detected in the blood samples of infected patients using RT-PCR. The presence of virus-specific antibodies in the serum of patients has been associated with viral clearance and protective immunity. However,

these are not widely used for the diagnosis of viral hepatitis as the role of HGV in causing hepatitis is still not clear.

Key points

- The hepatitis viruses include a range of unrelated human pathogens that specifically infect the liver.
- All the hepatitis viruses are RNA viruses, except the hepatitis B virus (HBV), which is a DNA virus belonging to the family Hepadnaviridae.
- The hepatitis A virus (HAV) is an enterovirus (type 72). It is worldwide in distribution and infection is by the feco-oral route.
- Antibody to HAV is protective, whereas cell-mediated immunity may contribute to pathology.
- Diagnosis is based on the demonstration of specific antibodies.
- Infection by HAV can be prevented by good personal hygiene and prevention of fecal contamination of drinking water and food.
- HBV is an enveloped, 42-nm spherical particle with a partially double-stranded DNA genome.
- Infection due to HBV is acquired through sexual intercourse, shared needles in drug use, perinatally from infected mothers to newborn infants or by transfusion of blood and blood products.
- HBV causes hepatitis B; it has also been implicated in hepatocellular carcinoma.
- For lab diagnosis, tests include detection in the serum of the surface antigen (HBsAg) and of IgM antibody to core antigen (anti-HBc); detection of envelope antigen itself (HBeAg) or antibody to HBeAg (anti-HBe) helps in the assessment of infectivity of the individual.
- Amplification of HBV DNA by PCR and isolation of the virus from blood or tissue are other diagnostic means.
- Screening of donated blood has greatly reduced the spread of HBV.
- Vaccination with cloned HBsAg has further reduced disease in some populations in developed countries. Antiviral therapies can help treat chronic infections.
- The hepatitis C virus (HCV) is an enveloped virus, 50–60 nm in diameter, with a genome of single-stranded RNA. Hepatitis C infection is one of the most common of all liver diseases, the infection being transmitted by contaminated blood products and by shared injections during drug use.
- HCV typically causes a chronic form of hepatitis. It has also been implicated in hepatocellular carcinoma.
- For diagnosis, antibodies to HCV antigen can be detected by enzyme immunoassay. The HCV RNA can be amplified by RT-PCR. Routine culture of HCV has not been established.

- The disease can be prevented by screening of blood and blood products prior to transfusion for the presence of anti-HCV antibody or HCV RNA.
- The hepatitis D virus (HDV) is an unusual, single-stranded, circular RNA virus that requires hepadnavirus helper functions for propagation in hepatocytes.
- The hepatitis E virus (HEV) causes enterically transmitted, non-A non-B hepatitis. It is a non-enveloped, single-stranded RNA virus.
- HEV is an important cause of large epidemics of acute hepatitis in the Indian subcontinent and elsewhere.
- Specific diagnostic tests for infection due to HEV include PCR to detect HEV RNA, and detection of both IgG and IgM anti-HEV antibodies.
- General measures for preventing HEV infection are similar to those for prevention of HAV infection.
- HGV RNA has been found in patients with acute, chronic and fulminant hepatitis, hemophiliacs, patients who had undergone multiple transfusions and hemodialysis, intravenous drug addicts and blood donors.

Questions

Essay

1. Enumerate the viruses affecting the liver. Discuss the pathogenesis and laboratory diagnosis of hepatitis B.

Short Answers

1. Viral markers of HBV
2. Laboratory diagnosis of HBV
3. Hepatitis B prophylaxis
4. Mutants of HBV
5. Hepatitis C virus

Part IV: Virology

Chapter 53

Retrovirus

Chapter navigation

- Human immunodeficiency virus (HIV)
  – HIV 1, 2 (causing AIDS)
- Simian AIDS
- Feline T lymphotropic virus (*Not pathogenic to humans*)

INTRODUCTION

The emergence and pandemic spread of the acquired immunodeficiency syndrome (AIDS) has posed the greatest challenge to public health in modern times.

History

The first indication of this new syndrome came in the summer of 1981, with reports from New York and Los Angeles (USA) of a sudden, unexplained outbreak of two very rare diseases, Kaposi's sarcoma and *Pneumocystis carinii* (*jirovecii*) pneumonia in young adults who were homosexuals or addicted to injected narcotics. This condition was given the name **acquired immune deficiency syndrome (AIDS).**

In **1983, Luc Montagnier** and his colleagues from the Pasteur Institute, Paris, isolated a retrovirus from a West African patient with persistent generalised lymphadenopathy and called it **lymphadenopathy-associated virus (LAV)**. In **1984, Robert Gallo** and his colleagues from the National Institute of Health, USA, reported the isolation of a retrovirus from AIDS patients and called it **human T cell lymphotropic virus III** or **HTLV III**. Retroviruses HLTV I and II had already been described in association with human T cell leukemia. International Committee on Virus Nomenclature in 1986 decided on the generic name **human immunodeficiency virus (HIV)** for these viruses.

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

HIV, the causative agent of AIDS, belongs to the **lentivirus** subgroup of the family **Retroviridae**. Besides HIV, related animal immunodeficiency viruses are also assigned to this group.

Members of the lentivirus group that cause immunodeficiency:

I. In primates

1. Human immunodeficiency viruses (HIV) types 1, 2

2. Simian immunodeficiency viruses (SIV) causing simian AIDS (SAIDS):
   a) Isolated from sooty mangabeys (SIV-SM) and from rhesus macaque (SIV-MAC); closely related to HIV type 2
   b) Isolated from chimpanzee (cpz)—closely related to HIV type 1

II. In non-primates

1. Feline T lymphotropic virus (FTLV) that causes feline AIDS (FAIDS)

Morphology

Envelope

HIV is a spherical, enveloped virus, about 90–120 nm in size (Fig. 53.1). The nucleocapsid has an outer icosahedral shell and an inner cone-shaped core, enclosing the ribonucleoproteins.

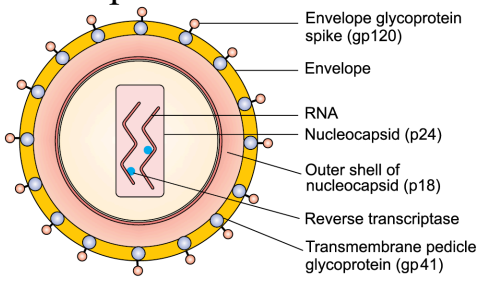


Fig. 53.1 Structure of HIV (diagrammatic representation)

Genome

The genome is composed **of two identical single-stranded, positive-sense RNA copies**, with the **reverse transcriptase enzyme** (a characteristic feature of retroviruses). When the virus infects a cell, the viral RNA is transcribed by the enzyme, first into single-stranded DNA and then to double-stranded DNA (**provirus)** which is integrated into the host cell chromosome. The provirus can remain latent for long periods, influencing host cell function. In response to viral promoters, the provirus initiates viral replication by directing the synthesis of viral RNA and other components.

Lipoprotein envelope

When the naked virus buds out through the host cell surface during viral replication, it acquires a lipoprotein envelope, which consists of lipid derived from the host cell membrane and glycoproteins coded by the virus. The major virus-coded envelope proteins are the projecting knob-like spikes on the surface and the anchoring transmembrane pedicles. The spikes constitute the main surface component of the virus, which binds to the CD4

receptors (along with co-receptors CXCR4 and CCR5) on susceptible host cells. Transmembrane pedicles cause cell fusion.

Viral Genes and Antigens

The HIV genome contains the three structural genes (*gag*, *pol* and *env*) as well as other non-structural and regulatory genes specific to the virus (Fig. 53.2). The products of these genes, both structural and non-structural, act as antigens. The detection of these antigens and their antibodies is useful for the diagnosis and prognosis of HIV infection.

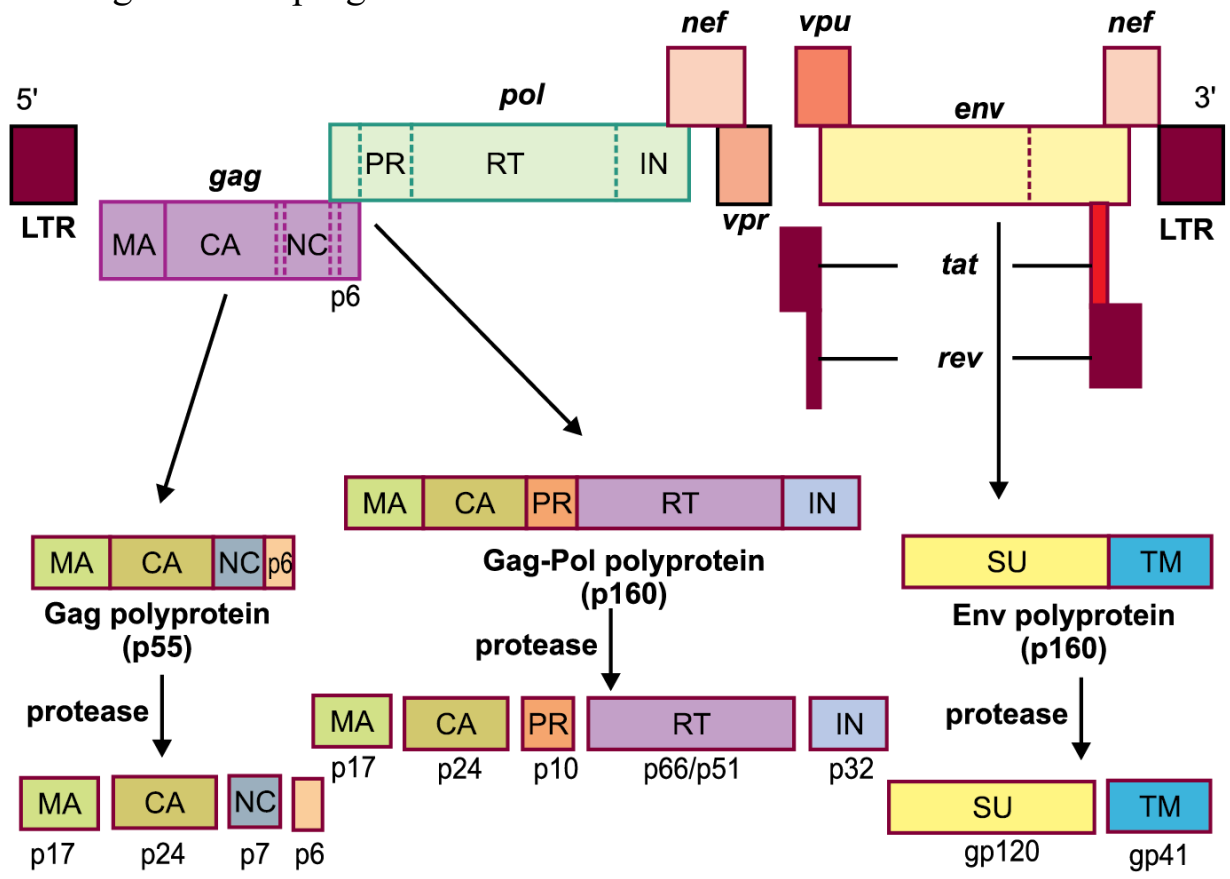


Fig. 53.2 HIV genome—diagrammatic representation

Major antigens of HIV

A. Envelope antigens
1. Spike antigen—gp120 (principal envelope antigen)
2. Transmembrane pedicle protein—gp41

B. Shell antigen
1. Nucleocapsid protein—p18

C. Core antigens

1. Principal core antigen—p24
2. Other core antigens—p15, p55

D. Polymerase antigens—p31, p51, p66

Genes coding for structural proteins

- The ***gag* gene** determines the core and shell of the virus. It is expressed as a precursor protein, p**55**. This precursor protein is cleaved into three proteins, p15, p18 and p24, which make up the viral core and shell. The **major core antigen is p24** which can be detected in serum during the early stages of HIV infection before antibodies appear. Decline of free anti-p24 antibody and reappearance of p24 antigen in circulation indicates exacerbation of the illness.
- The ***env* gene** determines the synthesis of the envelope glycoprotein gp160, which is cleaved into the two envelope components: gp120, which forms the surface spikes, and gp41, which is the transmembrane-anchoring protein. Antibodies to gp120 are present in circulation till the terminal stage of the infection.
- The ***pol* gene** codes for polymerase reverse transcriptase and other viral enzymes, such as protease and endonuclease. It is expressed as a precursor protein, which is cleaved into proteins p31, p51 and p66. They do not have much diagnostic or prognostic significance.

Non-structural and regulatory genes

- *tat* **(trans-activating gene)** enhances the expression of all viral genes.
- *nef* **(negative factor gene)** downregulates viral replication.
- *rev* (**regulator of virus gene**) enhances the expression of structural proteins.
- *vif* **(viral infectivity factor gene)** influences the infectivity of viral particles.
- *vpu* **(only in HIV-1)** and *vpx* (only in HIV-2) enhance the maturation and release of progeny viruses from cells (**detection of the type-specific sequences, *vpu* and *vpx*, is useful in distinguishing between infection by HIV-1 and -2**).
- *vpr* stimulates the promoter region of the virus.
- **LTR (long terminal repeat) sequences**, one at either end, contain the sequences that give the promoter, enhancer and integration signals.

Antigenic Variation and Diversity of HIV

HIV is a highly mutable virus, unlike HTLV. It exhibits frequent antigenic variation as well as differences in other features such as nucleotide sequences, cell tropism, growth characteristics and cytopathology. **Antigenic variation exists in isolates of HIV from different places or persons and also between sequential isolates from the same person**. This variability of HIV is believed to be due to the error-prone nature of reverse transcription.

HIV 1 and HIV 2

Based on molecular and antigenic differences, two types of HIV have been recognised. The original isolates of HIV and the related strains prevalent all over the world belong to HIV type 1. HIV-2, which was first isolated from West Africa in 1986, has only 40 per cent genetic similarity with HIV-1. It is more closely related to simian immunodeficiency virus than to HIV-1 and **is much less virulent than HIV-1**. It is largely confined to West Africa with a few reports from other areas, including western and southern India.

HIV-1 subtypes

- HIV-1 strains have been classified into at least **ten subtypes** based on sequence analyses of their *gag* and *env* genes. These subtypes are designated A to J.
- All the subtypes constitute Group M (for 'major'), which cause most of the HIV-1 infections worldwide.
- A few HIV-1 strains isolated from West Africa (Cameroon, Gabon) do not fall within Group M and have been designated Group O (for 'outlier').
- Some later isolates of HIV-1 from Cameroon, distinct from the M and O groups have been called Group N (for 'new'). All known HIV groups and subtypes are present in Cameroon, West Africa.

HIV-1 subtypes show geographical distribution, though this is often blurred by viral trafficking. Subtype A is the most prevalent worldwide, while B is the most common in the Americas and Europe. The common subtypes in Africa are A, C and D, while in Asia, the common subtypes are E, C and B. Type E is presently considered a recombination of A and E called the AE type (also known as CRFs or circulating recombinant forms). **In India and China, subtype C is the most prevalent**.

Antigenic differences between HIV strains may be important in serodiagnosis. Infection by HIV-1 or -2 may not be identified unless the

corresponding type is represented in the test antigen.

Transmissibility of subtypes The subtypes seem to vary in frequency of transmissibility by different routes. The subtypes common in Asia and Africa (C and E) are more readily transmitted by heterosexual contact than the American strains (subtype B) which are preferentially spread through blood—by injection and homosexual contact.

Growth Characteristics

Differences in growth characteristics are sometimes observed between HIV isolates from asymptomatic carriers (grow slowly) and those from AIDS patients (grow faster). Variations may account for differences in the clinical course of HIV-infected persons.

Susceptibility to Temperature

HIV is thermolabile, being inactivated in 10 minutes at 60°C and in seconds at 100°C. At room temperature (20–25°C), in dried blood, it may survive for up to seven days. On autopsy, HIV has been isolated from various tissues up to 16 days after death. It withstands lyophilisation. The virus, in lyophilised blood products, can be inactivated by heating at 68°C for 72 hours and in liquid plasma at 60°C for 10 hours.

HIV is inactivated in 10 minutes by treatment with 50% ethanol, 35% isopropanol, 0.5% lysol, 0.5% paraformaldehyde, 0.3% hydrogen peroxide, 1% nonidet p40 or 10% household bleach. It is also inactivated at the extremes of (pH 1 and 13). Bleaching powder and household bleach are effective for surface decontamination. The standard recommendation is a hypochlorite solution at a concentration of 0.5% available chlorine (5 g/L; 5,000 ppm). For the treatment of contaminated medical instruments, a 2% solution of glutaraldehyde is useful.

Pathogenesis

Infection is acquired when the virus enters the blood or tissues of a person and comes into contact with a suitable host cell, principally the CD4 lymphocyte.

Cell receptors for virus attachment

The receptor for the virus is any cell bearing a **CD4 antigen,** primarily the $CD4^+$ (helper/inducer) T lymphocyte. About 5–10 per cent of B lymphocytes and 10–20 per cent of monocytes and macrophages, including alveolar macrophages in the lungs and Langerhans cells in the dermis, are

susceptible. Glial cells and microglia in the central nervous system are also susceptible. **Follicular dendritic cells from the tonsils can be infected by HIV without the involvement of CD4.**

Specific binding of the virus to the CD4 receptor is by the envelope glycoprotein gp120. Cell fusion is essential for infection to take place. This is brought about by transmembrane gp 41 (Fig. 53.3). Binding to the CD4 receptor requires the participation of a co-receptor molecule, which has been identified as CXCR4 for
T cell-tropic HIV strains and CCR5 for macrophage-tropic strains.

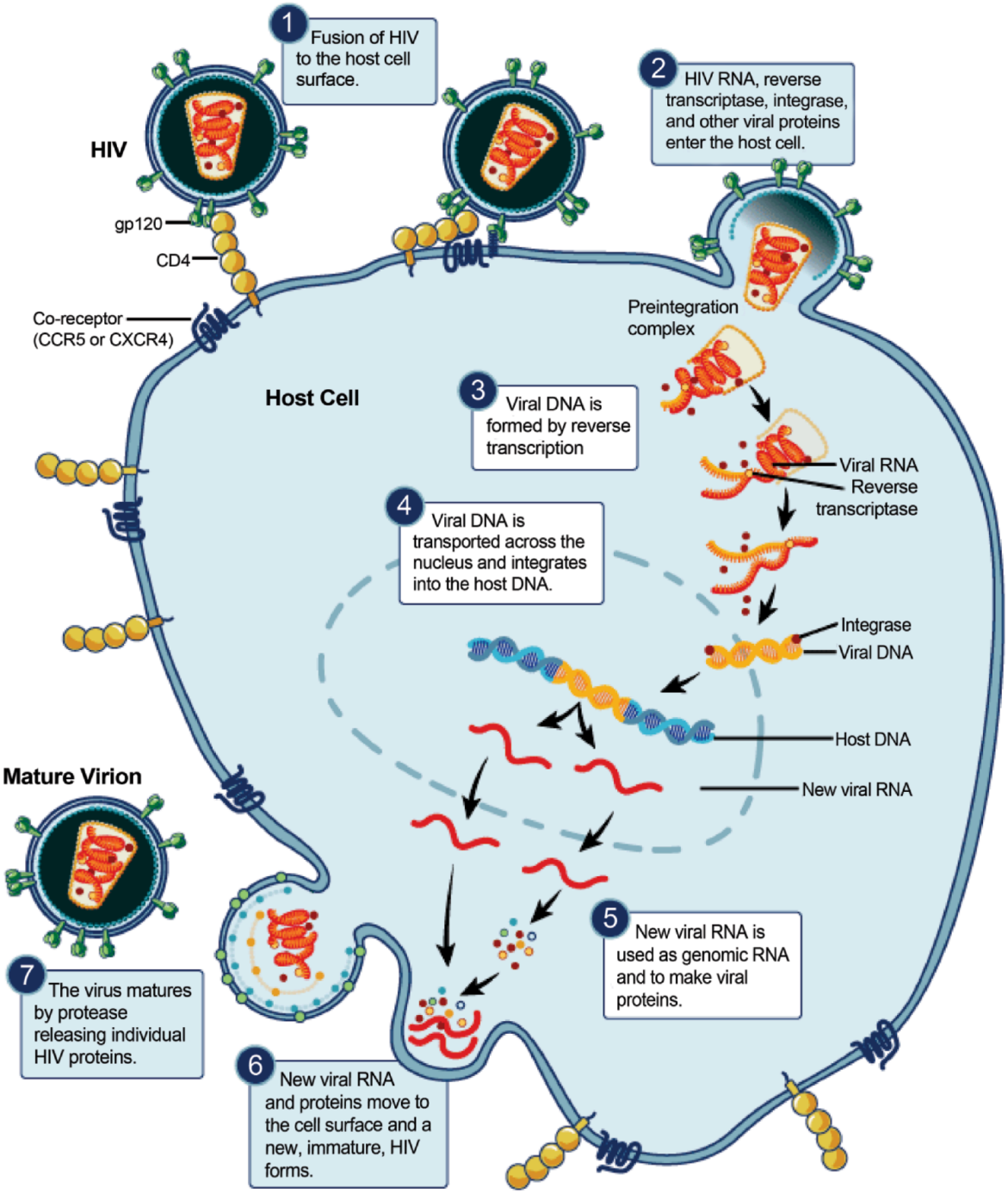

1
Fusion of HIV to the host cell surface.
HIV
gp120
CD4
Co-receptor (CCR5 or CXCR4)
2
HIV RNA, reverse transcriptase, integrase, and other viral proteins enter the host cell.
Preintegration complex
Host Cell
3
Viral DNA is formed by reverse transcription
Viral RNA
Reverse transcriptase
4
Viral DNA is transported across the nucleus and integrates into the host DNA.
Integrase
Viral DNA
Host DNA
New viral RNA
Mature Virion
5
New viral RNA is used as genomic RNA and to make viral proteins.
7
The virus matures by protease releasing individual HIV proteins.
6
New viral RNA and proteins move to the cell surface and a new, immature, HIV forms.

Fig. 53.3 Replication of HIV in a susceptible cell with CXCR4 and CCR5 receptors (*Source:* Public Health Image Library, ID 18162 / National Institute of Allergy and Infectious Diseases (NIAID) / CDC)

Replication

After fusion of the virus with the host cell membrane, the HIV genome is uncoated and internalised into the cell. Viral reverse transcriptase mediates

the transcription of HIV RNA into double-stranded DNA, which is integrated into the genome of the infected cell through the action of the viral enzyme integrase, causing a latent infection. The long and variable incubation period of HIV infection is because of the latency. In an infected individual, HIV can be isolated from the blood, lymphocytes, cell-free plasma, semen, cervical secretions, saliva, tears, urine and breast milk.

The **primary pathogenic mechanism** in HIV infection is the damage caused to the $CD4^+$ T lymphocyte. The T4 cells decrease in number with the reversal of T4:T8 (helper:suppressor) cell ratio. Infected T4 cells do not release normal amounts of interleukin-2, gamma interferon and other lymphokines, suppressing cell-mediated immune response.

Though the main damage is to cellular immunity, humoral mechanisms are also affected. AIDS patients are unable to respond to new antigens. An important feature in HIV infection is the polyclonal activation of B lymphocytes, leading to hypergammaglobulinemia of all classes of immunoglobulins, particularly IgG and IgA. In infants and children, IgM levels are also elevated. The hypergammaglobulinemia may also be responsible for allergic reactions due to immune complexes (type 3 hypersensitivity).

Monocyte–macrophage function is also affected, apparently due to the lack of secretion of activating factors by the T4 lymphocytes. As a result, chemotaxis, antigen presentation and intracellular killing by monocytes/macrophages are diminished. The activity of NK cells and cytotoxic T lymphocytes is also affected.

The principal immunological abnormalities seen in HIV infection are listed in the following box.

Immunological abnormalities in HIV infection

I. Features that characterise AIDS

1. Lymphopenia
2. Selective T cell deficiency—reduction in the number of T4 (CD4) cells, inversion of T4:T8 ratio
3. Decreased delayed hypersensitivity on skin testing
4. Hypergammaglobulinemia—predominantly IgG and IgA; IgM also in children
5. Polyclonal activation of B cells and increased spontaneous secretion of Ig

II. Other consistently observed features

1. Decreased in vitro lymphocyte proliferative response to mitogens and antigens
2. Decreased cytotoxic response by T cells and NK cells

3. Decreased antibody response to new antigens
4. Altered monocyte/macrophage function
5. Elevated levels of immune complexes in serum

Clinical manifestations in HIV infections are not primarily due to viral cytopathology but are secondary to the failure of immune response. This renders the patient susceptible to opportunistic infections and malignancies. Dementia and other degenerative neurological lesions are due to the action of the virus on the central nervous system (CNS) cells.

Epidemiology

HIV-1 M, O and **N types** may represent independent transmissions from chimpanzees to humans. The source of HIV-2 has been established as SIV from the sooty mangabey monkey, *Cercocebus atys.*

Transmission

The virus has spread globally, with geographically different prevalence rates. HIV is spread only by three modes (Table 53.1):

Table 53.1 Common modes of transmission of HIV and their relative risk

Types of exposure	Mode of transmission	Approximate chance of infection per exposure
I	Sexual intercourse: anal, vaginal, oral	0.1–1.0%
II	Blood and blood products, Factor VII, etc., blood transfusion	>90%
III	Tissue and organ donation: semen, cornea, bone marrow, kidney, etc.	50–90%
IV	Injections and injuries: needle sharing by drug addicts Injections with unsterile syringes and needles Needle-stick and other injuries in health staff Surgical wounds	0.5–1.0%
V	Mother to baby: Transplacental At birth	30%

	After birth Breast milk	

- Sexual contact with infected persons (heterosexual or homosexual)
- Through blood and blood products
- From infected mothers to babies (intrapartum, perinatal, postnatal)

There is no evidence of HIV transmission by other means including casual contact or through insects.

HIV is primarily a **sexually transmitted infection**. In the West, homosexual and bisexual men are infected more often than heterosexuals. However, in Africa and Asia, men and women are equally affected. Transmission in the developing countries is almost always heterosexual and can take place in both partners.

The best method of checking sexual transmission and other high-risk activities for infection is through counselling and health education.

The second mode of transmission is **through blood and blood products**. Screening of blood donors is now mandatory, which must include p24 antigen screening.

Contaminated needles can transmit the infection. This is particularly relevant in drug addicts who share syringes and needles. Higher incidence has been detected in the northeastern states of India besides some other parts of the country. The use of **unsterile syringes and needles** by health workers makes iatrogenic infection likely. The use of disposable syringes, needles and other equipment has reduced the incidence.

The risk of **needle-stick injury** is a concern for health care personnel, though the chances of infection are much less than with the hepatitis B virus. Transmission of infection from **mother to child** can take place before, during or after birth. Early infant diagnosis has now been introduced by NACO to detect HIV infection in newborns. This is done by **testing for pro-viral DNA by PCR** on blood collected from the newborn by heel prick. This is absorbed on dry filter paper. This dried blood spot is sent to a referral laboratory for testing, and the baby is treated. HIV may be present in breast milk and may be transmitted through breastfeeding.

HIV is not transmitted by social and domestic contact, shaking hands, hugging, putting cheeks together or dry kissing.

ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS)

Clinical Case

A 35-year-old commercial sex worker presented to the medical OPD with history of weight loss, decreased appetite and cough with expectoration for one month. Her total WBC count was low, sputum AFB was positive and graded 2+; HIV serology was reactive with the three-test strategy of the National AIDS Control Organisation (NACO). CD4 count was 350 cells/mL. The patient was referred to the ART centre for antiretroviral and antitubercular chemotherapy. She was advised to return for a follow-up after two weeks and to use a condom during sexual contact.

Clinical Features of HIV Infection

AIDS is the last stage in the wide spectrum of clinical features in HIV infection. The Centers for Disease Control and Prevention, USA, have classified the clinical course of HIV infection under various groups (Table 53.2).

The natural course of HIV infection passes through the following stages:

Table 53.2 Classification system for HIV infection
(Centers for Disease Control and Prevention, USA)

Categories	Clinical presentation
Group I	Acute HIV syndrome
Group II	Asymptomatic infection
Group III	Persistent generalised lymphadenopathy
Group IV	Other diseases
Subgroup A	Constitutional disease—AIDS-related complex (ARC)
Subgroup B	Neurologic diseases
Subgroup C	Secondary infectious diseases
Subgroup C1	Specified infectious diseases listed in the CDC surveillance definition for AIDS such as *P. carinii* pneumonia, cryptosporidiosis, toxoplasmosis, generalised strongyloidiasis, cryptococcosis, CMV or herpes infections
Category C2	Other specified secondary diseases, such as oral hairy leukoplakia, salmonella bacteremia,

	nocardiosis, tuberculosis, thrush
Subgroup D	Secondary cancers, such as Kaposi's sarcoma, lymphomas
Subgroup E	Other conditions

Group I—acute HIV infection

Within 3–6 weeks of infection with HIV, about 50 per cent of persons experience low-grade fever, malaise, headache, lymphadenopathy, sometimes with rash and arthropathy resembling glandular fever. Rarely, there may be acute encephalopathy. Spontaneous resolution occurs within weeks.

- **Seroconversion illness** Tests for HIV antibodies are usually negative at the onset of the illness but become positive during its course, though in many of those infected, there may not be any apparent clinical illness.
- **Acute retroviral syndrome** Patients with this syndrome may have fever, fatigue, rash, pharyngitis or other symptoms. HIV antigenemia (p24 antigen) can be demonstrated at the beginning of this phase.

Group II—asymptomatic or latent infection

All persons infected with HIV, whether or not they experience seroconversion illness, pass through a phase of symptomless infection (**clinical latency**) which may last up to several years. They are positive for HIV antibody and are infectious. The median time between primary HIV infection and the development of AIDS has been stated as approximately 10 years. This period of clinical latency, however, does not mean viral latency since virus multiplication goes on throughout.

The infection progresses over time through various stages:
CD4 lymphocytopenia → Minor opportunistic infections → Persistent generalised lymphadenopathy (PGL) → AIDS-related complex (ARC) → Full-blown AIDS

- **Long-term survivors** or **long-term non-progressors** About 5–10 per cent of the infected appear to escape clinical AIDS for 15 years or more. The mechanisms for such prolonged survival are

not clear, though many viral and host determinants may be responsible.

> The virus load in the plasma is of prognostic value. Viral killing of cells goes on throughout the illness. The steady state of the virus (**virus set point**) in a patient varies with individuals. High set point correlates with rapid disease progression.

The $CD4^+$ T cell count decreases steadily, from over 1,000 per microlitre to about 500 or less in the stage of acute infection. When the count falls to <200, clinical AIDS usually sets in. Hence **case definition by CDC includes all HIV-infected cases with $CD4^+$ T cell counts of 200 or less, irrespective of their clinical condition** (*Clinical case*).

Group III—persistent generalised lymphadenopathy (PGL)

This has been defined as the presence of enlarged lymph nodes in two or more non-contiguous extrainguinal sites that persist for at least three months in the absence of any current illness or medication that may cause lymphadenopathy. Such cases may progress to ARC or AIDS.

Group IV—AIDS-related complex (ARC)

The typical constitutional symptoms are fatigue, unexplained fever, persistent diarrhea and marked weight loss (‘**diarrhea and dwindling**’) of more than 10 per cent of the body weight.

Generalised lymphadenopathy and splenomegaly are usually present. ARC patients are usually severely ill and many of them progress to AIDS in a few months.

AIDS This is the **end-stage disease**, representing the irreversible breakdown of immune defence mechanisms, leaving the patient open to progressive opportunistic infections and malignancies.

The clinical severity of AIDS varies with the type of infection or malignancy present. In early AIDS, many patients are ill only during episodes of infection, which may respond to treatment. Between episodes, they may be relatively well and able to resume normal life. Patients with Kaposi’s sarcoma are less ill than those with other malignancies. The illness progresses inexorably and death ensues in months or years.

Opportunistic Infections in AIDS

Opportunistic infections in the various organ systems and other common complications due to AIDS (Table 53.3) include the following:

Table 53.3 Major opportunistic infections and malignancies commonly associated with untreated AIDS patients

Etiology	Specific agents/infections
Parasitic	1. Toxoplasmosis 2. Cryptosporidiosis 3. Isosporiasis 4. Generalised strongyloidiasis
Mycotic	1. *Pneumocystis jirovecii* infection 2. Candidosis 3. Cryptococcosis 4. Aspergillosis 5. Histoplasmosis
Bacterial	1. Mycobacterial infections—tuberculosis and non-tuberculous infections 2. Salmonellosis 3. Campylobacter infection 4. Nocardia and actinomycetes 5. Legionellosis
Viral	1. CMV 2. Herpes simplex
Malignancies	1. Kaposi sarcoma 2. Lymphomas—Hodgkin and non-Hodgkin types

Respiratory system The commonest presentation is **dry cough, dyspnea and fever**. In developing countries including India, the most important pathogen is
M. tuberculosis, with increasing incidence of multidrug-resistant strains. A double epidemic of HIV and drug-resistant tuberculosis is the current challenge in developing countries. Pneumonia may be viral (CMV) or fungal [*Pneumocystis jirovecii* (Fig. 53.4), *Cryptococcus* or *Histoplasma*].

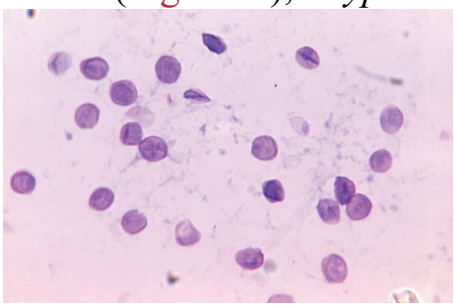


Fig. 53.4 *Pneumocystis jirovecii* in lung tissue stained with methenamine silver stain (*Source:* Public Health Image Library, ID 2744 / CDC)

Gastrointestinal system Oral and esophageal thrush (candidiasis) (Fig. 53.5a), herpetic stomatitis, gingivitis, hairy leukoplakia or Kaposi's sarcoma are common oral manifestations. Dysphagia may be due to esophageal candidosis. Cryptosporidium, salmonellae, atypical mycobacteria, isospora, CMV or adenoviruses frequently cause intestinal infections. Disseminated strongyloidiasis may also occur.

'Gay bowel syndrome' is chronic colitis commonly seen in male homosexuals. Amoeba, giardia and a host of diarrheagenic bacteria have been reported to be responsible for this condition.

Central nervous system The typical CNS opportunistic infections are toxoplasmosis and cryptococcosis. Infections are also seen with CMV, herpes simplex, papovaviruses, mycobacteria, *Aspergillus*, *Cryptococcus* and *Candida*. Lymphomas of the central nervous system are common.

Malignancies Kaposi's sarcoma, Hodgkin's lymphoma and other non-Hodgkin's lymphomas are associated with AIDS.

Cutaneous diseases Herpes lesions, candidosis, xeroderma, seborrheic dermatitis, prurigo, folliculitis, impetigo and molluscum contagiosum are the common cutaneous lesions besides Kaposi's sarcoma (Fig. 53.5b).

Fig. 53.5 (a) Oral candidiasis and (b) Kaposi's sarcoma on the heels of an AIDS patient (*Source:* (a) Public Health Image Library, ID 6067 / John Molinari, Ph.D., University of Detroit, Detroit, Michigan; Sol Silverman, Jr., D.D.S. / CDC and (b) Public Health Image Library, ID 14430 / CDC)

Ophthalmic diseases Most common abnormal findings are cotton wool spots on the retina due to microvascular disease, seen on funduscopic

examination. CMV retinitis is the most devastating complication. Others could be herpes simplex and varicella-zoster retinitis.

Dementia HIV may cause direct cytopathogenic damage in the central nervous system. It can cross the blood–brain barrier and cause encephalopathy, leading to the loss of higher functions, progressing to dementia.

Wasting syndrome Generalised wasting is an AIDS-defining feature with >10% involuntary weight loss, fever, chronic diarrhea and fatigue.

Pediatric AIDS About a third to half the number of babies born to infected mothers are infected with HIV. Virus transmission to the fetus may occur as early as the first trimester, but infection is more common perinatally.

Many of the infected children do not survive the first year of life. Children may also acquire the infection from blood transfusion or blood products.

Differences between adult and pediatric AIDS

- Children develop humoral immunodeficiency early, leading to recurrent bacterial infections.
- They fail to thrive.
- Chronic diarrhea is more common among children.
- Lymphadenopathy is more pronounced.
- Tuberculosis and opportunistic bacterial infections are common manifestations in pediatric AIDS.
- Lymphocytic interstitial pneumonia is seen mostly in children.
- Kaposi's sarcoma, toxoplasmosis and cryptococcosis are less common in children than in adults.

Laboratory Confirmation of HIV and AIDS

Laboratory tests are done for:

i) Diagnosis of clinically suspected cases
ii) Monitoring of treatment
iii) Screening of blood
iv) Antenatal screening of mothers
v) Early infant diagnosis
vi) Screening high-risk groups

Methods

Tests depend on the stage of the disease. Principally, three methods can be employed:

i) Viral isolation

ii) Detection of antibody to various antigens of the virus
iii) Detection of viral DNA, RNA or antigens

Policy of National Aids Control Organisation (NACO), Government of India
To bring about a reliable set of standardised tests in India, the **National AIDS Control Organisation (NACO),** Government of India, is credited for implementing a strategy for testing different categories of suspected individuals exposed to HIV. To ensure quality and uniformity in reporting the incidence and prevalence of the disease, a set of guidelines have been provided for conducting serological tests and their interpretation. It has

been made mandatory for all testing laboratories to follow these guidelines. A **pre-** and **post-test counselling** must be conducted to educate the patient. Further, no test can be carried out without the prior consent of the patient.

Methods for the detection of HIV infection

1. Antigen detection

Following a single massive infection, as by blood transfusion, the viral antigens may be detectable in blood after about two weeks. The major core antigen, p24, is the earliest virus marker to appear in blood, hence is tested for early diagnosis. (The IgM antibodies appear in about 4–6 weeks, followed by IgG antibodies Fig. 53.6a and b.)

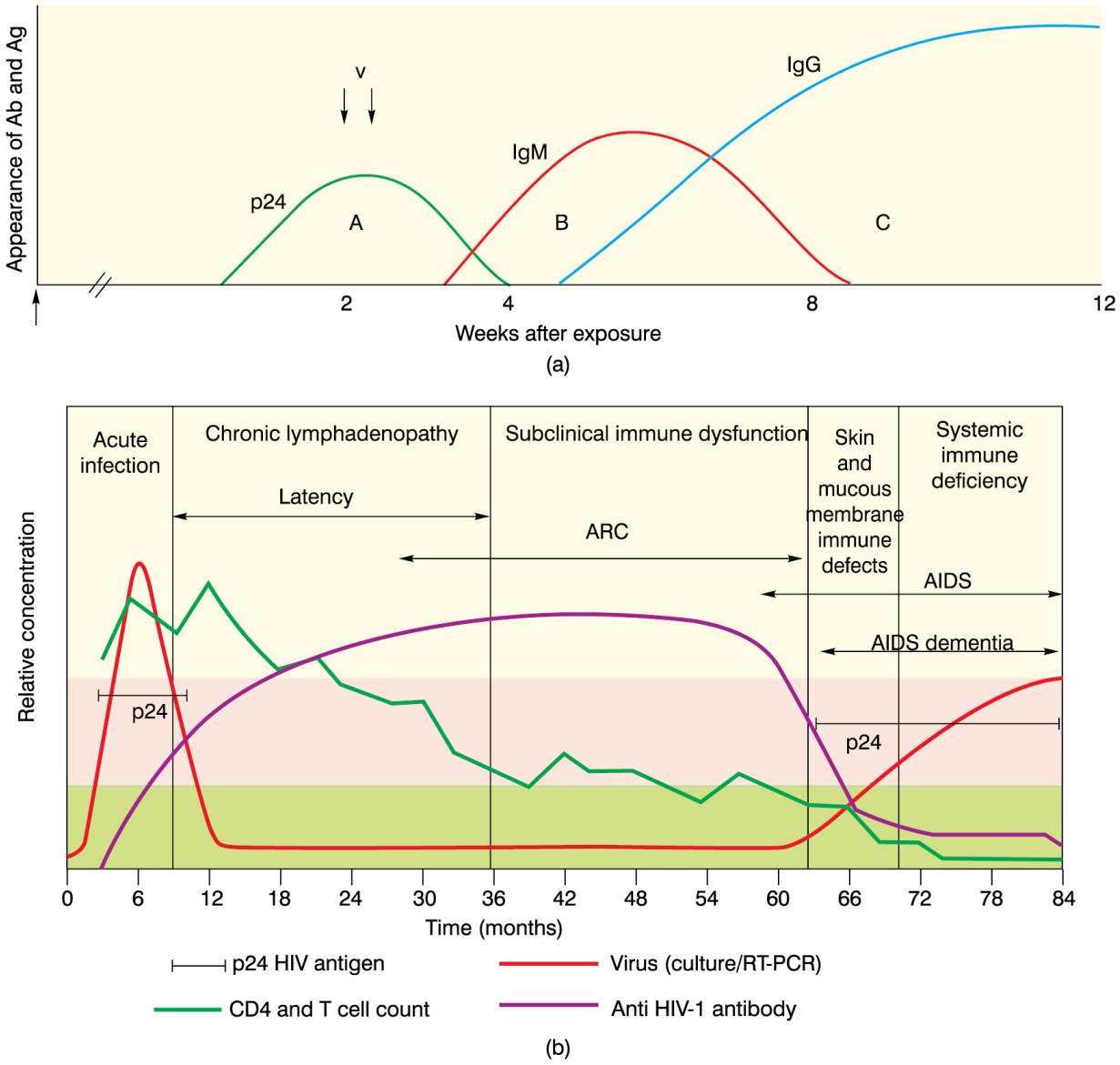


Fig. 53.6 (a) Sequence of appearance of p24 antigen and antibodies after a massive HIV infection and (b) illustration of the usual time-course of immune response, viremia, and disease resulting from untreated HIV-1 infection.

p24 capture assay

Antibody-bound p24 antigen may be demonstrable after dissociation. The p24 antigen capture assay (ELISA), which uses

anti-p24 antibody as the solid phase, can be used for this. The test is positive in about 30 per cent of HIV-infected persons. With prior dissociation of the antigen–antibody complex, the positive rate increases to about 50 per cent. The test is most useful in persons recently exposed to risk of infection, in whom the antibody test is negative in the first few weeks of infection and in the terminal phase of the illness.

Seroconversion

This refers to the appearance of IgM antibody in the patient's serum following the initial period of p24 antigenemia and viremia. Later, free p24 antigen disappears from circulation and remains absent during the long asymptomatic phase, to reappear only when severe clinical disease sets in.

2. Virus isolation

Once infected with HIV, a person remains infected for life. The virus is present in circulation in body fluids, within lymphocytes or is cell-free. Virus titres parallel p24 titres.

The virus isolation is done in containment laboratories. HIV is isolated from infected persons from the peripheral lymphocytes by **co-cultivation.** The patient's lymphocytes are cultivated with uninfected healthy lymphocytes, in the presence of interleukin-2.

3. Detection of viral nucleic acids

Amplification of viral DNA and RNA are the most sensitive and specific tests. They can be detected by DNA PCR, RNA PCR (RT-PCR) and bDNA assay.

- ***DNA PCR*** Peripheral lymphocytes from the subject are lysed and the proviral DNA is amplified using primer pairs from relatively constant regions of the HIV genome (from the *gag* and LTR regions). The amplified DNA is detected by probes based on nucleic acid hybridisation. The test can detect HIV proviral DNA at a frequency of one copy per 10,000 cells.
- ***RT-PCR (Reverse transcriptase PCR)*** This is a useful test to detect the disease progression and monitor response to therapy.
- ***bDNA (branched DNA) assay*** Sequential oligonucleotide hybridisation steps are used to amplify the viral RNA.

Dried blood spots on filter paper may be used as an alternative to plasma. This is adopted to transport specimens to molecular testing facilities from remote places in developing countries. In India, dried blood spots are used for **early infant diagnosis as a part of the prevention of parent-to-child transmission (PPTCT).**

4. Antibody detection

Demonstration of antibodies is the simplest and most widely employed

technique for the diagnosis of HIV infection. However, it may take **2–8 weeks** to months for antibodies for appear. **This seronegative infective stage is known as the window period**. The individual may be highly infectious during this period. Hence antibody testing is not totally dependable for detecting infectious persons, e.g., from among blood donors. **Infection can be detected during the window period by p24 antigen assay**.

IgM antibodies disappear in 8–10 weeks while IgG antibodies remain throughout. When immunodeficiency becomes severe following clinical AIDS, some components of anti-HIV antibody (anti-p24) may disappear.

Anti-HIV antibody tests are used for either screening or confirming the infection (Table 53.4).

Table 53.4 Serological markers detected during various phases of HIV infection

State of infection	Antigens/antibodies	Anti-HIV IgM	Anti-HIV IgG	Western blot pattern
Early infection	+ (p24 antigen)	–	–	–
Acute (seroconversion)	+ → –	– → +	– → +	+
Partial illness	gp120, gp41, gp160 antibodies gp120	+ → –	+	+
Carriers and asymptomatic individuals	P24 ag – and gp120, gp41, gp160 antibodies +	–	+	+
PGL	P24 antibodies –	–	+	+
AIDS	P24, P17 and P55 antibodies decline	–	+	+

Tests used for screening

These tests are highly sensitive, have a broad spectrum of reactivity, are simple to perform and can be automated to handle large numbers of samples at a time. As they are not highly specific, they may give a few false positive results. All sera testing positive on a screening test are to be confirmed before the sample is declared reactive.

ELISA

Indirect ELISA is the method most commonly used. HIV grown in

continuous T lymphocyte cell line or obtained by recombinant techniques is the source of the antigen. It includes groups and subtypes of HIV-1 and HIV-2. The antigen is coated on microtitre wells or other suitable solid phases. The test serum is added, and if the antibody is present, it binds to the antigen. After washing the excess unbound antibodies, antihuman immunoglobulin linked to a suitable enzyme is added, followed by a colour-forming substrate. If the test serum contains anti-HIV antibody, a photometrically detectable colour is formed, which can be read by the ELISA reader.

ELISA is simple and relatively inexpensive but false positive reactions are not uncommon, particularly with sera containing rheumatoid factor, anti-lymphocyte or other autoantibodies and in hepatic disease.

Modifications of ELISA in which the antibody in the test serum either competes with enzyme-conjugated anti-HIV antibody or is captured by antihuman immunoglobulin onto a solid phase are more specific. Capture ELISA specific for IgM antibody is also available. Immunometric assays are highly sensitive and specific.

While ELISA is ideal for screening several serum samples at a time, it is inconvenient for testing single samples quickly.

Rapid tests

A number of 'rapid tests' have been introduced for this purpose, such as:

- Cassette ELISA
- Immunochromatographic tests (ICT)
- Coated particle agglutination
- Immunoperoxidase or dip-stick tests

Specimens used for tests are:

- Blood from finger-prick
- Dried blood on filter paper
- Saliva and urine

Confirmatory or supplemental tests

These are performed on serum samples that are reactive in screening tests.

The confirmatory tests may also be needed to resolve discordant results of two or more rapid or ELISA tests.

Western blot test This is the most commonly used confirmatory test (Fig. 53.7).

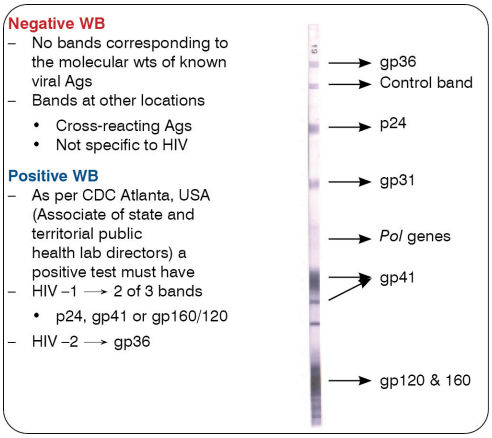

Negative WB
– No bands corresponding to the molecular wts of known viral Ags
– Bands at other locations
• Cross-reacting Ags
• Not specific to HIV
Positive WB
– As per CDC Atlanta, USA (Associate of state and territorial public health lab directors) a positive test must have
– HIV –1 → 2 of 3 bands
• p24, gp41 or gp160/120
– HIV –2 → gp36
gp36
Control band
p24
gp31
Pol genes
gp41
gp120 & 160

Fig. 53.7 Western blot test for HIV antibody

Procedure HIV proteins, separated according to their electrophoretic mobility (and molecular weight) by polyacrylamide gel electrophoresis, are blotted onto strips of nitrocellulose paper. The patient's sera are reacted with these proteins on the strips followed by an enzyme-conjugated antihuman globulin. A suitable substrate is then added, which produces a distinct colour band where the specific antibody has reacted with the blotted viral protein.

Results The position of the band on the strip indicates the antigen with which the antibody has reacted. In a positive serum, multiple proteins' bands are seen. Mainly, the following bands are noted:

- **p24** (*gag* gene, core protein)
- **p31** (*pol* gene, reverse transcriptase)
- **gp41**, **gp120** or **gp160** (*env* gene, surface antigens)

A **positive reaction with proteins representing three genes is conclusive**. The test may be considered positive if it also shows bands against at least two of the following gene products: p24, gp41 and gp120/160. Western blot is a useful confirmatory test but the interpretation remains subjective and demands considerable experience. In indeterminate cases, the Western blot may be repeated after a specified period of time. It

may be necessary to do a p24 assay in indeterminate results.

Serological diagnosis markers of infection may not be reliable in AIDS as antibody formation may be affected by the immune deficiency.

Line immunoassays (LIAs) These are based on the application of recombinant and synthetic peptide antigens on a plastic support strip in a manner similar to the immunoblot assay. LIAs are second- or third-generation assays and have the potential to be used as supplemental tests. Combination assays and differentiating infection by HIV-1 and HIV-2 can also be done in LIA.

Reasons for HIV screening

- Epidemiological surveillance using unlinked anonymous HIV testing
- Transfusion and transplant safety
- Diagnosis of HIV infection in symptomatic and asymptomatic individuals
- Prevention of parent-to-child transmission (including early infant diagnosis)
- For post-exposure prophylaxis (PEP)
- Research

Strategies for HIV testing

In India, the **National AIDS Control Organisation (NACO)** of the Ministry of Health, Government of India, has laid out guidelines and strategies for screening, testing and monitoring HIV infected/suspected individuals.

ELISA/Rapid tests/Supplemental tests (E/R/S) are used in strategies I, II and III.

Supplemental tests are employed in cases of indeterminate/discordant result of E/R/S.

Strategies for testing different categories of samples as per NACO guidelines

(*Source:* National Guidelines for HIV Testing, July 2015; National AIDS Control Organisation, Ministry of Health & Family Welfare, Government of India)

Strategy I This strategy is used to **screen blood/blood products, organs, tissues, sperm,** etc. The sample is subjected once to E/R for HIV (Fig. 53.8).

NACO recommends the use of ELISA kits with a sensitivity of ≥99.5 per

cent and specificity of ≥98 per cent and rapid kits with a sensitivity of ≥99.5 per cent and specificity of ≥98 per cent. If negative, the specimen is considered free of HIV. Such a sample is considered fit for donating.

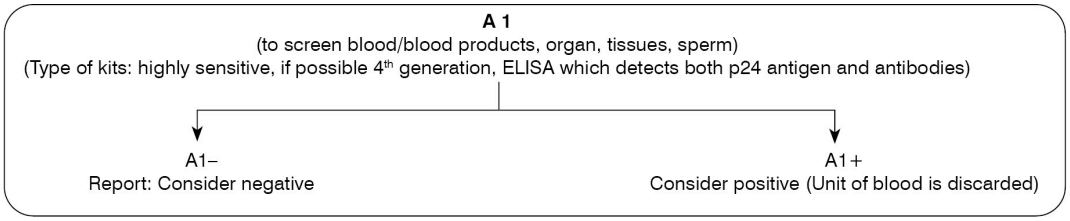

A 1
(to screen blood/blood products, organ, tissues, sperm)
(Type of kits: highly sensitive, if possible 4th generation, ELISA which detects both p24 antigen and antibodies)
A1–
Report: Consider negative
A1+
Consider positive (Unit of blood is discarded)

Fig. 53.8 Interpretation of strategy I tests

Strategy II This strategy is used for **surveillance** (IIA) and **diagnosis** (IIB) in cases where some AIDS indicator disease is present (Figs. 53.9 and 53.10). If a serum sample is positive in the first ELISA, it is subjected to a second ELISA, which utilises a system different from the first one. If the second ELISA is positive, it is reported as positive. If it is negative (the second ELISA), the result is considered negative.

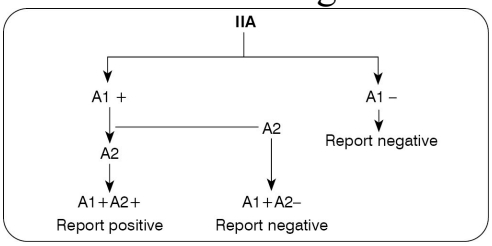


Fig. 53.9 Strategy IIA (for surveillance—anonymous and unlinked—2 tests are required)

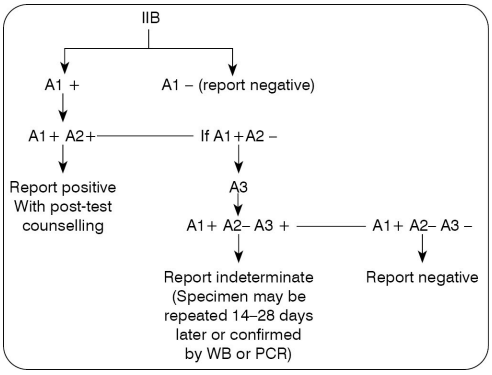


Fig. 53.10 Strategy IIB (for diagnosis of symptomatic or high-risk individuals)

Strategy IIB This strategy is used to determine the HIV status of clinically symptomatic suspected AIDS cases in which blood/serum/plasma is tested with highly sensitive screening and confirmatory tests based on different

principles and/or antigens as compared to the first test.

Strategy III This is used in asymptomatic individuals. It is similar to strategy II, with the added third positive ELISA test being required for a sample to be reported HIV-reactive. In this, the first ELISA is the one with the highest sensitivity and the second and third ELISAs have the highest specificity (Fig. 53.11).

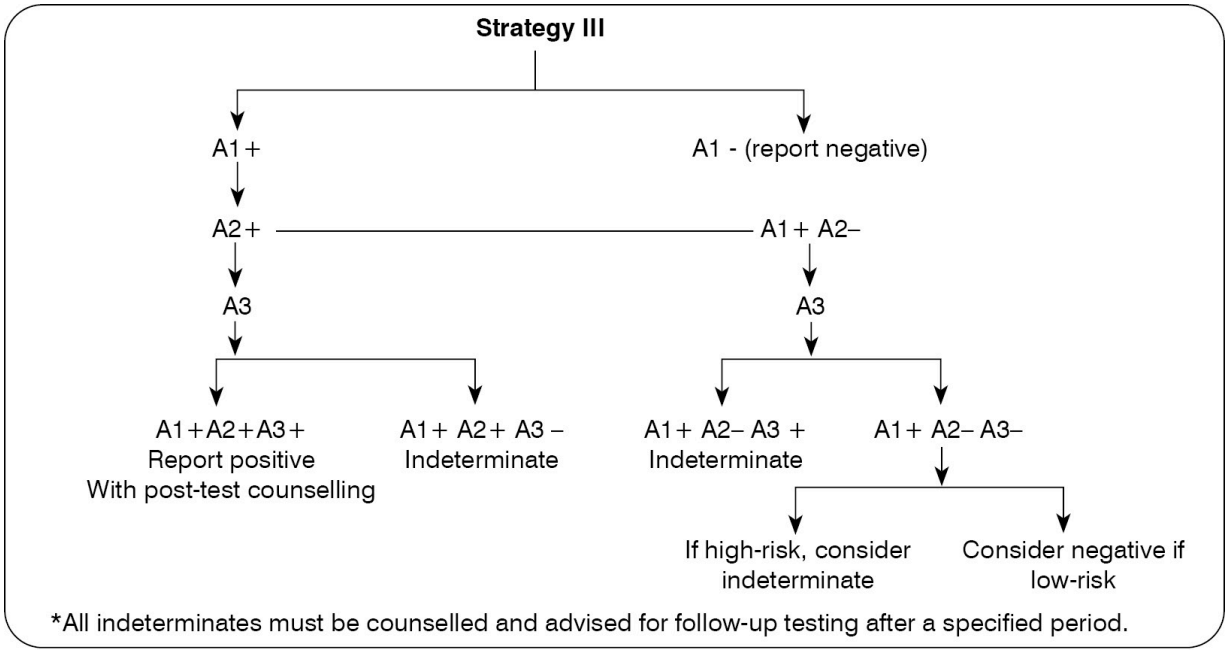


Fig. 53.11 Strategy III (To detect HIV infection in asymptomatic individuals presenting to ICTC or PPTCT)

Early infant diagnosis

Infants born to HIV positive mothers have to be screened early after birth in order to start treatment or monitor for mother-to-child transmission. The strategy to diagnose in infants/children below 18 months is by DNA polymerase chain reaction (DNA PCR), which detects HIV-1 pro-viral DNA integrated to human genome. This is done by collecting either whole blood or spots of the infant's blood dried on filter paper (DBS). The blood is eluted out of the filter paper and the sample is used to extract the DNA for PCR. Antibodies are not detected in infants below 6 months of age, as the maternal antibody would give a false positive test.

Applications of serological tests

Serological tests for HIV infection are used in the following situations:

1. ***Screening*** Populations or selected target groups are screened for epidemiological purposes. Screening of entire populations is neither

feasible nor practicable. However, screening of a target population is useful. It is mandatory for all donors of blood, blood products, semen, cells, tissues and organs to be screened. Screening for the p24 antigen can detect those in the window period also. As the infection can be transmitted from mother to baby before, during or after birth, screening of all antenatal women has been made mandatory.

2. ***Seroepidemiology*** Antibody surveys have been most useful in identifying the geographical extent of HIV infection and in other epidemiological studies such as the spread of the infection from identified sources.
3. ***Diagnosis*** Serology is almost always positive in persons with clinical features of AIDS. It may, however, be negative in acute illness and sometimes in the very late cases where the immune system is completely suppressed. Routine serology may also be negative in HIV-2 infections, which are likely to be missed if antibody testing is done with the HIV-1 antigen alone.

Although HIV-1 and HIV-2 are related, there are important structural differences between them. Accurate diagnosis and differentiation of HIV-1 and HIV-2 is crucial for treatment, as HIV-2 is intrinsically resistant to NNRTI (non-nucleoside reverse transcriptase inhibitors), the pillar of national first-line ART regimen.

Antibody testing may also help to check whether infection has taken place following an exposure, such as sexual contact, blood transfusion or needle-stick injury. Serology after two months and, if negative, after six months, would be sufficient. If serology is negative six months after exposure, infection is unlikely to have occurred.

Avidity assay may be done to assess the binding between IgG antibodies and the corresponding HIV antigen. This binding increases over time in newly acquired infections. The strength of the binding reflects the duration of infection and helps in differentiating between recent infections and chronic ones.

Prognosis

In a person infected with HIV, loss of detectable anti-p24 antibody indicates clinical deterioration. This is also associated with HIV antigenemia and increased virus titre in circulation.

Non-specific or immunological tests

The following parameters indicate immunodeficiency in HIV infection:

- Total leucocyte and lymphocyte count to demonstrate leucopenia (count usually below 2,000/mm^3)
- T cell subset assays—absolute $CD4^+$ T cell count is usually less than 200/mm^3; T4:T8 cell ratio is reversed
- Thrombocytopenia (low platelet count)
- Raised IgG and IgA levels
- Diminished CMI as indicated by skin tests
- Lymph node biopsy shows abnormalities

Laboratory Monitoring of HIV Infection

Some laboratory tests are important in monitoring the course of HIV infection.

1. **$CD4^+$ T cell count,** which reflects the current immunological competence of the patient, is most often used to monitor the course. A count below 500 indicates disease progression and the need for antiretroviral therapy. Counts below 200 denote risk of serious infection.
2. **Viral load—**it is necessary to monitor HIV RNA during the course of treatment. This is usually done by two methods, RT-PCR and bDNA assay.
3. **Beta-2-microglobulin** and **neopterin** are two substances that have a predictive value on the progression of HIV disease as they rise with advancing disease.

Prophylaxis

Prevention of AIDS depends on general measures such as health education, identification of sources and decrease in high-risk behaviour. **No specific vaccine is available**. The high mutability, diverse antigenic types and subtypes, long latency and persistence as provirus in infected cells pose several problems in the development of vaccines.

Vaccine research

Several possible strategies have been explored for vaccine production. These include:

- **Immunisation** of experimental animals like chimpanzees and monkeys with modified whole virus
- Subunits, based on envelope glycoproteins expressed in animal cells, bacteria, viruses—or as synthetic epitopes on adjuvant carriers
- Target cell protection by anti-CD4 antibody or genetically engineered CD4; a number of candidate vaccines are being tested in clinical trials in humans

Post-exposure prophylaxis

This refers to the comprehensive management given to minimise the risk of infection following potential exposure to bloodborne pathogens, such as HIV.

'**Exposure**' [expressed as **exposure code (EC)**] for risk of developing bloodborne infections is defined as:

- Percutaneous injury (needle-stick injury)
- Contact with mucous membrane of eye or mouth
- Contact with non-intact skin
- Contact with intact skin when the duration of contact is prolonged with blood or other potentially infectious body fluids

Although the risk of HIV transmission by these routes is less than 1%, PEP is recommended within 72 hours of exposure, depending on the exposure category of HCP (health care personnel) and the HIV status code of the patient (Figs. 53.12 and 53.13).

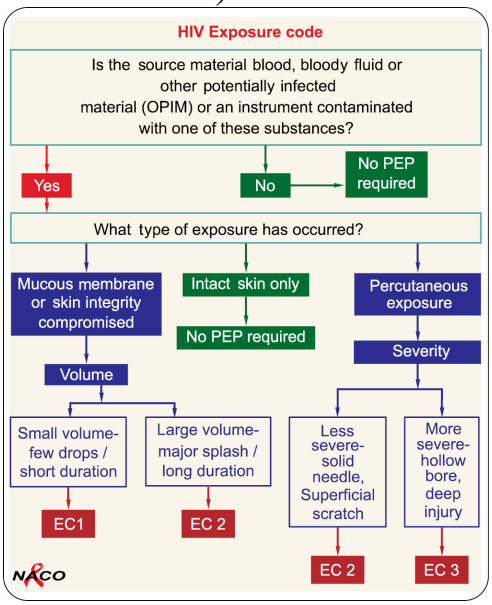


Fig. 53.12 Guidelines of National AIDS Control Organisation (NACO)

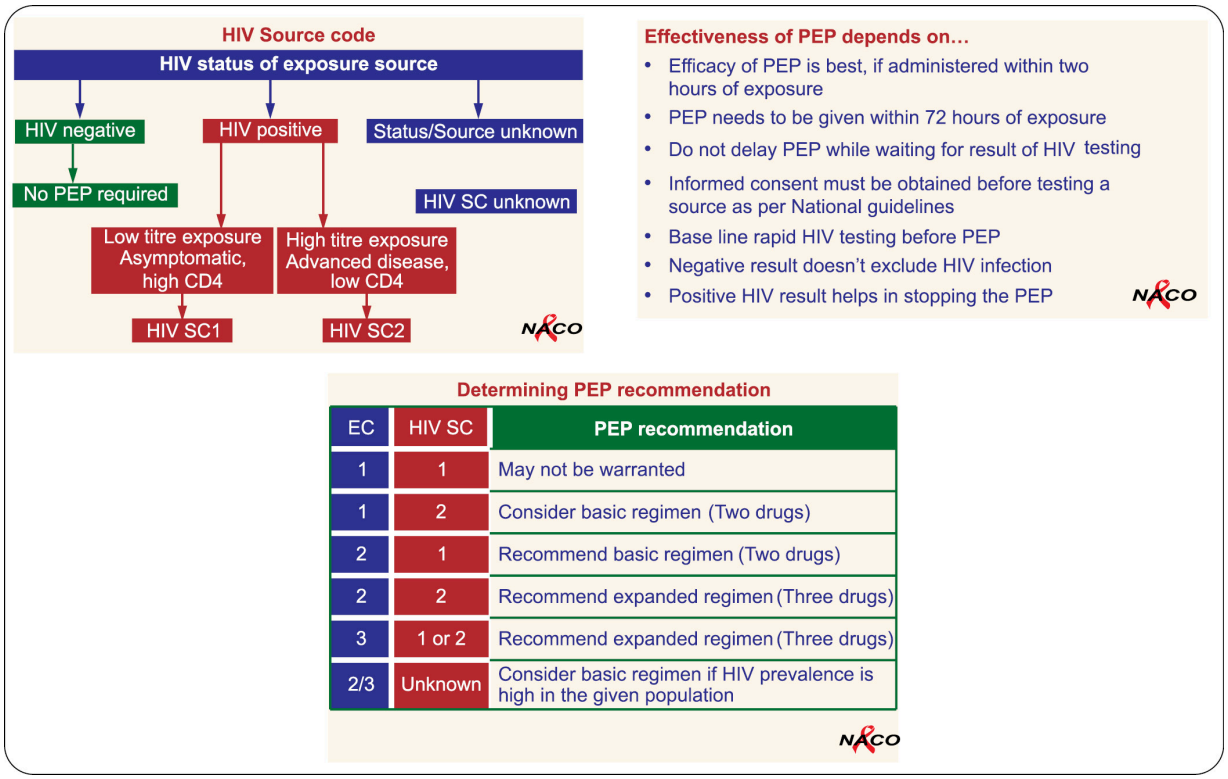

HIV Source code
HIV status of exposure source
HIV negative
HIV positive
Status/Source unknown
No PEP required
HIV SC unknown
Low titre exposure
Asymptomatic,
high CD4
High titre exposure
Advanced disease,
low CD4
HIV SC1
HIV SC2
NACO
Effectiveness of PEP depends on...
Efficacy of PEP is best, if administered within two hours of exposure
PEP needs to be given within 72 hours of exposure
Do not delay PEP while waiting for result of HIV testing
Informed consent must be obtained before testing a source as per National guidelines
Base line rapid HIV testing before PEP
Negative result doesn't exclude HIV infection
Positive HIV result helps in stopping the PEP
NACO
Determining PEP recommendation
EC | HIV SC | PEP recommendation
1 | 1 | May not be warranted
1 | 2 | Consider basic regimen (Two drugs)
2 | 1 | Recommend basic regimen (Two drugs)
2 | 2 | Recommend expanded regimen (Three drugs)
3 | 1 or 2 | Recommend expanded regimen (Three drugs)
2/3 | Unknown | Consider basic regimen if HIV prevalence is high in the given population
NACO

Fig. 53.13 Recommendations for post-exposure prophylaxis (PEP)

Management of AIDS

The approaches to the treatment of AIDS include:

i) The treatment and prophylaxis of infections and tumours
ii) General management
iii) Immunorestorative measures
iv) Specific anti-HIV agents

Prompt diagnosis, counselling and appropriate treatment of opportunistic infections and tumours in the early stage of AIDS can be very useful and the patient may be able to resume normal life in-between episodes of illness. General management of the patient requires the understanding and cooperation of the health staff in the hospital and of relatives at home. Fears about imaginary risks have to be allayed and reassurance given that the patient can be kept at home or treated in the hospital without danger to contacts if proper precautions are taken.

Antiretroviral treatment

Highly active antiretroviral treatment **(HAART)** is the mainstay of

treatment. It leads to complete suppression of plasma viremia; opportunistic infection rates are decreased and the quality of life for people living with HIV and AIDS **(PLHA)** is improved. Adherence to treatment can delay the development of drug resistance and the need for second-line treatment. Specific treatment with antiretroviral drugs is the mainstay in the management of HIV infection. A number of effective drugs have become available in recent years (Table 53.5). The sites of action of major antiretroviral drugs are shown in Fig. 53.14.

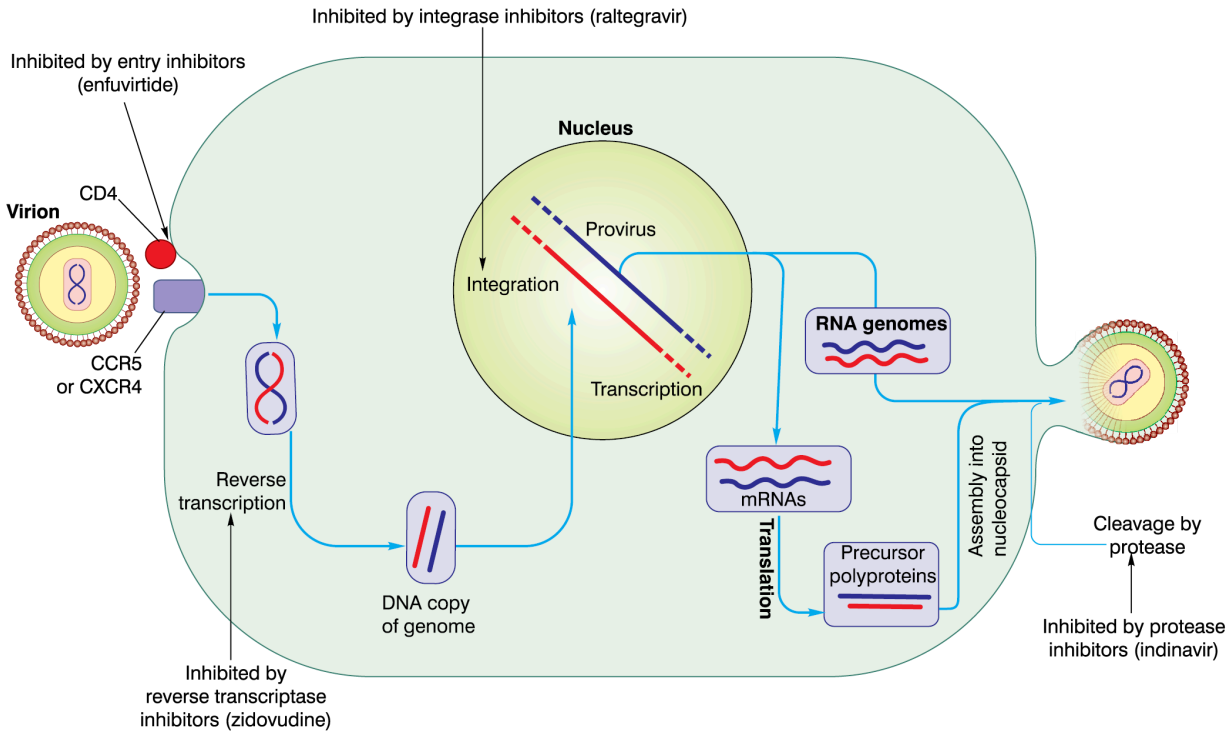


Fig. 53.14 Site of action of anti-retroviral drugs

Table 53.5 Anti-retroviral drugs

Mechanism of action	Drugs
Entry inhibitors i) Fusion inhibitor ii) Co-receptor antagonist	 Enfuvirtide Maraviroc
Integrase inhibitor	Raltegravir Dolutegravir
Nucleoside reverse transcriptase inhibitors (NRTI) Non-nucleoside inhibitors (NNRTI)	Abacavir, zidovudine (azidothymidine (AZT)) didanosine, zalcitabine, lamivudine

	Nevirapine, etravirine, efavirenz
Protease inhibitors	Saquinavir, ritonavir, indinavir

The antiretroviral drugs include nucleoside analogues like zidovudine (azidothymidine, AZT), didanosine, zalcitabine, lamivudine and protease inhibitors like saquinavir, ritonavir and indinavir. These drugs have been used as monotherapy or in various combinations. However, adverse reactions and their high cost restrict their wide use in resource-poor countries.

The National AIDS Control Organisation (NACO) provides free anti-retroviral treatment through several ART centres across India.

Steps for immunorestorative therapy such as administration of interleukin-2, thymic factors, leucocyte transfusion and bone marrow transplantation have not been very helpful.

Vaccines

Problems with designing an HIV vaccine are (i) the nature of the high frequency of mutation of the virus, (ii) the integration of the viral DNA into the host cell's genome, whereby the virus may remain in the latent form for years and (iii) the lack of understanding of the mechanism of protective immunity. The other is that, some infected persons are non-progressors and some remain uninfected despite being exposed several times. Despite these problems, several vaccine trials have been undertaken using gp120 in animals and human volunteers but these have failed to provide protection. A recent vaccine using poxvirus vector with several viral proteins was used followed by a booster dose with an envelope protein of the HIV. This provided only 30% protection. Till date, no safe and effective vaccine against HIV infection has been released.

Key points

- Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are the causative agents of acquired immunodeficiency syndrome (AIDS) worldwide. HIV is about 90–120 nm in size, spherical in shape, icosahedral in symmetry and enveloped.
- There are ten subtypes, A through J, which fall into group M, other groups being O and N.
- The virus is easily inactivated by many disinfectants.

- Important structural components of the virus include the surface antigen gp120, the transmembrane antigen gp41, the matrix protein p17 and the capsid antigen p24. The three important enzymes contained in the virion are reverse transcriptase, protease and integrase.
- There are two copies of single-stranded RNA. Two genes, *gag* and *pol*, code for the formation of reverse transcriptase, protease and integrase, and for p17 and p25. Another gene, *env*, codes for the formation of gp120 and gp41.
- AIDS is a late manifestation of HIV disease. Symptoms are varied, from asymptomatic to flu-like symptoms to tumours (Kaposi's sarcoma, lymphomas, anal and cervical carcinoma) and opportunistic infections. The disease is spread mainly by sexual intercourse, through blood and from the mother to the fetus or newborn.
- In India, the National AIDS Control Organisation (NACO), Government of India, is responsible for implementing government policy on the control of AIDS and HIV infection. Laboratory diagnosis includes antigen detection, virus isolation, PCR and antibody detection.
- The mainstay of the diagnosis of HIV infection is serological tests: ELISA is the most frequently used method for screening blood samples for anti-HIV antibody. The Western blot assay is the gold standard for diagnosis, and seropositivity is diagnosed when antibodies against the *Env* and *Gag* proteins are detected.
    - HIV antigen can be detected as early as three weeks in the course of HIV infection, and before the appearance of antibodies.
    - Isolation of the virus is accomplished by co-cultivation of the patient's lymphocytes with fresh peripheral blood cells of healthy donors or with suitable culture lines.
- HIV RNA can be demonstrated by probes or by RT-PCR techniques. The latter are particularly useful since the viral RNA can be detected as early as 72 hours after infection, thus establishing diagnosis, and the response to therapy can be assessed.
- There are three strategies for HIV testing as per NACO, which are based on the type of setting where the testing is done. Screening tests like ELISA/simple/rapid tests (E/R/S) are used in strategies I, II and III. Supplemental or confirmatory tests are done in cases of indeterminate/discordant results of E/R.
- Prevention of AIDS depends on changes in human behaviour. Treatment of AIDS depends on antiviral medication and prevention and treatment of opportunistic infections and tumours.

Questions

Essay

1. Enumerate the sexually transmitted infections and write the laboratory diagnosis of HIV.

Short Answers

1. HIV viral genes and antigens

2. Antigenic variations of HIV
3. Pathogenesis of AIDS
4. Laboratory diagnosis of HIV infection
5. Significance of viral markers in HIV infection
6. Western blot test and interpretation for diagnosis of HIV infection
7. NACO strategies for HIV testing in India
8. Prevention of HIV
9. Opportunistic infections in AIDS patients
10. Rapid tests for HIV
11. Role of PCR in the diagnosis of HIV
12. Monitoring of patient on ART

Part IV: Virology

CHAPTER 54

Oncogenic Viruses and Other Miscellaneous Viruses

Chapter navigation

- Oncogenic DNA and RNA viruses
- Miscellaneous virus
  - Human papillomavirus
  - Parvovirus
  - Coronavirus
  - Arenavirus
  - Ebola virus

ONCOGENIC VIRUSES

Viruses that produce tumours in their natural hosts or in experimental animals or induce malignant transformation of cells on culture are known as oncogenic viruses.

History

The association of viruses with malignancy was described by **Rous** in 1911 when he showed that a solid malignant tumour, fowl sarcoma, was caused by a virus—a discovery for which he was awarded the **Nobel Prize** belatedly in 1966.

Viruses that cause tumours in animals were first demonstrated by Shope, who isolated the rabbit fibroma virus in 1932 and the **papillomavirus** in 1933. Considerable interest was aroused by the discovery by Stewart and Eddy (1957) of the **polyomavirus,** which could produce a wide variety of neoplasms when injected into newborn rodents. Injection of certain types of human **adenovirus** into newborn hamsters was shown by Trentin (1962) to

cause sarcomas. Burkitt (1963) identified a peculiar geographical distribution of lymphoma in African children. The **Epstein–Barr virus** isolated from Burkitt's lymphoma has been identified as the causative agent.

However, it is now acknowledged that virus infections account for 10–20 per cent of human malignancies. These include **hepatocellular carcinoma** caused by hepatitis B and C viruses, **cervical cancer** by certain types of papillomaviruses, anaplastic **nasopharyngeal carcinomas** by the EB virus and adult cutaneous **T cell lymphoma/leukemia** by HTLV-1.

Transformation

This represents the various changes that accompany the conversion of a normal cell into a malignant cell (by oncogenic viruses) (Table 54.1). This is a multistep process and may be partial or complete. For example, some viral agents can '**immortalise**' infected cells, so that they become capable of continuous multiplication in culture, without possessing other features of malignancy. Transformation is recognised primarily by a change in the morphology of cultured cells. **Transformed cells are altered in shape and lose the property of 'contact inhibition' so that, instead of growing as a monolayer, they grow piled up, one over another, forming 'microtumours'**. Foci of transformation can be discerned easily and are used in the assay of oncogenic viruses such as the Rous sarcoma virus.

Table 54.1 Properties of cells transformed by viruses

Class	Properties of transformed cells
I	Altered cell morphology: Fibroblasts become shorter, parallel orientation is lost, chromosomal aberrations appear
II	Altered cell metabolism: Increased growth rate, increased production of organic acids and acid mucopolysaccharides
III	Altered growth characteristics: Loss of contact inhibition, formation of heaped-up growths (microtumours), capacity to divide indefinitely in serial culture, capacity to grow in suspension or in semisolid agar
IV	Antigenic alterations: Appearance of new virus-specified antigens (T antigen–TSTA), loss of surface antigens, cells become agglutinable by lectins

V	Capacity to induce tumours in susceptible animals

About a quarter of the 600 or so animal viruses possess an oncogenic potential (Table 54.2). The viruses associated with cancers in human beings are presented in Table 54.3. Both RNA and DNA viruses are oncogenic.

Table 54.2 List of oncogenic viruses

RNA Viruses
I. Retroviruses
1. Avian leukosis viruses
2.Murine leukosis viruses
3. Murine mammary tumour virus
4. Leukosis-sarcoma virus of various animals
5. Human T cell leukemia viruses
DNA Viruses
I. Papovaviruses
1. Papillomaviruses of human beings, rabbits and other animals
2. Polyomavirus
3. Simian virus 40
4. BK and JC viruses
II. Poxviruses
1. Molluscum contagiosum
2. Yaba virus
3. Shope fibroma
III. Adenoviruses
Many human and non-human types
IV. Herpesviruses
1.Marek's disease virus
2. Lucke's frog tumour virus
3. Herpesvirus pan, papio, ateles and saimiri
4. Epstein–Barr (EB) virus
5. Herpes simplex virus (HSV) types 1 and 2

6. Cytomegalovirus
V. Hepatitis B and C viruses

Table 54.3 Viruses associated with human cancer

Virus family	Virus	Types of cancer
Papovaviridae	Human papillomavirus	Cervical, vulvar, penile cancers Squamous cell carcinoma
Herpesviridae	EB virus	Nasopharyngeal carcinoma African Burkitt's lymphoma
	HSV type 2	B cell lymphoma, cervical carcinoma
Hepadnaviridae	Hepatitis B virus	Hepatocellular carcinoma
Flaviviridae	Hepatitis C virus	
Retroviridaee	HTL virus	Adult T cell leukemia

All oncogenic **RNA viruses** belong to a single family: Retrovirus. Retroviruses are responsible for naturally occurring leukemia and sarcoma in several species of animals. Among **DNA viruses**, some herpesviruses and hepadnaviruses cause malignant tumours in their natural hosts.

ONCOGENIC DNA VIRUSES

Papovaviruses

Human papillomavirus (HPV) The association between human papillomavirus (HPV) infection and cancer of the cervix uteri, particularly HPV types 16 and 18, has been established. A vaccine for this is now available.

The continuous cell line HeLa, derived many decades ago from a cervical carcinoma and used widely in various laboratories, has been found to contain HPV-18 DNA.

Polyomavirus The BK and JC viruses, which cause widespread asymptomatic human infection, can induce tumours in immunodeficient subjects.

Simian virus 40 (SV40) Transformation is induced in cultured cells from several species, including human cells.

Poxvirus

Three members of the poxvirus group induce benign tumours: rabbit fibroma, molluscum contagiosum and Yaba virus. Similar tumours can be induced experimentally in many species of primates, including human beings. The tumours regress spontaneously in a few weeks.

Adenovirus

Though some types (12, 19, 21) may produce sarcomas in newborn rodents after experimental inoculation, they do not appear to have any association with human cancer.

Herpesvirus

Many herpesviruses have been associated with natural cancers in animals and humans.

Marek's disease

This is a fatal contagious neurolymphomatosis of chickens. No infectious virus particle can be isolated from the lesions or seen under the electron microscope. Marek's disease can be prevented by a live avirulent vaccine. This is the first instance of a malignant disease being controlled by a viral vaccine.

Lucke's tumour of frogs

A herpesvirus is considered to be the causative agent of a renal adenocarcinoma in frogs.

Herpesvirus saimiri

This virus was isolated from a culture of squirrel monkey kidney cells. It causes fatal lymphoma or reticulum cell sarcoma when injected into owls, monkeys or rabbits.

Epstein–Barr virus

This virus is regularly found in cultured lymphocytes from **Burkitt's lymphoma** patients. In the body, the tumour cells contain no virus but cell lines established from them contain 5–20 per cent of cells that produce the virus. The virus multiplies only in human lymphoid cell lines. Serological

surveys show that infection with the virus is worldwide. Infection is usually asymptomatic. In young adults without pre-existing antibodies, EB virus infection induces **infectious mononucleosis**. Lymphoma is believed to occur when the infection takes place in children whose immune systems are compromised, as for instance, by chronic malaria. EB virus-associated lymphomas have been reported in transplant recipients. EBV has also been linked to nasopharyngeal carcinoma in the Chinese male population in southeast Asia and East Africa.

Herpes simplex virus

An association has been proposed (though not proved) between herpes simplex type 2 infection and cancer of the uterine cervix. It has also been suggested that herpes simplex type 1 infection may be associated with cancer of the lip. Herpesvirus type 8 has been linked to Kaposi's sarcoma.

Cytomegalovirus

This has been associated with carcinoma of the prostate and Kaposi's sarcoma.

Hepatitis B Virus

HBV has been claimed to be directly or indirectly involved in the causation of **hepatocellular carcinoma**. Studies in many countries have demonstrated an excess prevalence of markers of HBV infection in patients with primary hepatocellular carcinoma as compared to matched controls or with the general population. Hepatitis C virus infection has also been reported to lead to hepatocellular carcinoma.

ONCOGENIC RNA VIRUSES

Retrovirus

Retroviruses are enveloped, spherical viruses that are released by budding through the host cell membrane. They are approximately 100 nm in size. Their genome consists of two identical, linear, single-stranded RNA molecules. The icosahedral nucleocapsid core encloses the helical ribonucleoprotein and is surrounded by an envelope composed of glycoprotein and lipid.

The characteristic feature of retroviruses is the presence within the virion of the unusual enzyme **RNA-dependent DNA polymerase** or **reverse transcriptase** (hence the name *retro*, meaning 'reverse'). Unlike the classical transcription of genetic information from DNA to RNA, the reverse transcriptase enzyme prepares a DNA copy of the retroviral RNA genome—initially an RNA:DNA hybrid and then its double-stranded DNA form, called the **provirus**, which is integrated into the DNA of the infected host cell. It is from the provirus that all retrovirus proteins are translated. Infection with oncogenic retroviruses does not lead to cytolysis or death of infected cells but the provirus remains integrated with host cell DNA for the rest of the life of the cell.

Classification

While all oncogenic RNA viruses belong to the family Retroviridae, all retroviruses are not oncogenic. The family Retroviridae is classified into three subfamilies:

1. Oncornvirinae, comprising all oncogenic RNA viruses (formerly called oncornavirus)
2. Spumavirinae, containing the non-oncogenic 'foamy viruses' (*spuma* = foam) causing asymptomatic infection in several animal species
3. Lentivirinae, including the viruses causing 'slow infections' (*lentus* = slow) in animals, as well as the human and related animal immunodeficiency viruses

Types based on host range

Retroviruses are widely distributed, being found in nearly all vertebrates, including animals, birds and reptiles. Based on the host range and types of disease caused, oncogenic retroviruses can be considered under the following groups:

- ***Avian leukosis complex*** This is a group of antigenically related viruses which induce avian leukosis (lymphomatosis, myeloblastosis and erythroblastosis viruses) or sarcoma in fowls [Rous sarcoma virus (RSV)].
- ***Murine leukosis viruses*** This group consists of several strains of murine leukemia and sarcoma viruses, named after the

investigators who first described them (e.g., Gross, Friend, Moloney and Rauscher).

- ***Mammary tumour virus of mice*** This virus occurs in certain strains of mice that have a high natural incidence of breast cancer.
- ***Leukosis-sarcoma viruses of other animals*** A number of viruses have been isolated from leukosis and sarcomas in various species of animals: cat, hamster, rat, guinea pig and monkey.
- ***Human T cell leukemia (lymphotropic) viruses (HTLV)*** These were isolated in 1980 from cell cultures from adult patients with cutaneous T cell lymphoma (mycosis fungoides) and leukemia (Sezary syndrome) in the USA. Similar viruses have been isolated from patients with adult T cell leukemia in Japan and the Caribbean. HTLV type I is present worldwide but the disease is limited to endemic areas. Besides adult T cell leukemia, HTLV-I has also been associated with tropical spastic paraparesis, a demyelinating disease. The virus preferentially infects T4 (CD4) cells. Infected T cells express large quantities of interleukin-2 receptors. The closely related HTLV-II is also associated with T cell malignancy. HTLV infection is known to be spread through blood transfusions and other methods of transfer of leucocytes.

Host specificity

Retroviruses usually infect only one host species, the specificity being conditioned mainly by the presence of viral receptors on the host cell surface. Depending on their ability to grow in cells from different species, retroviruses have been classified as:

- **Ecotropic** (multiplying in cells of native host species only)
- **Amphotropic** (multiplying in cells of native and foreign species)
- **Xenotropic** (multiplying only in cells of foreign species but not of native host species)

Virus transmission

Two types of retrovirus transmission occur:

- **Exogenous retroviruses** are spread horizontally. Most oncogenic retroviruses are exogenous.
- **Endogenous retroviruses** are transmitted vertically from parent to offspring by the provirus integrated with the germline cell genome. The endogenous retrovirus provirus behaves like a cellular gene and is subject to regulatory control by the host cell. Endogenous retroviruses are usually silent and do not transform cells or cause any disease. They can be detected either by 'activation' after exposure to radiation or chemicals, or by nucleic acid hybridisation techniques.

Resistance

Retroviruses are labile, being inactivated at 56°C in 30 minutes, by mild acids, ether and formalin. They are stable at –30°C.

Antigens

Two types of antigens are present:

1. Type-specific glycoprotein antigens on the envelope
2. Group-specific nucleoprotein antigens in the virion core

Cross-reactions do not occur between surface antigens of retroviruses from different host species.

Genomic structure

Retroviruses have a relatively simple genomic structure (Fig. 54.1).

The provirus of a standard retrovirus (such as a non-defective avian or murine leukemia virus) consists of three genes required for viral replication: ***gag***, ***pol***, and ***env*** in that order from the 5′ to the 3′ end.

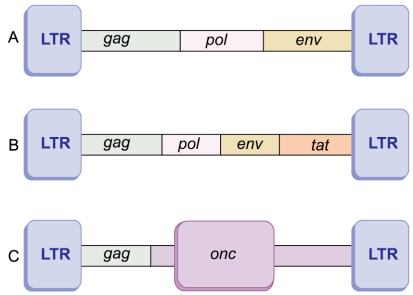


Fig. 54.1 Provirus genomic structure of different types of retroviruses; A. basic retrovirus

genome; avian leukemia viruses, slow transforming viruses; B. transregulating retroviruses HTLV, HIV; C. acute transforming retrovirus; oncogene replacing part of basic genome; replication defective

- The *gag* gene codes for the nucleocapsid core proteins which are group-specific antigens (hence the name).
- The *pol* gene encodes the RNA-dependent DNA polymerase.
- The *env* gene encodes the envelope glycoproteins. Long terminal repeat (LTR) sequences are present at either end of the provirus and linked directly to the host DNA.

LTRs exert regulatory control on provirus gene functions.

Some retroviruses (transregulating viruses) such as HTLV and HIV carry a fourth gene**, *tex*** or ***tat*,** after the *env* gene. This is a transactivating gene that regulates the function of viral genes.

Virus transformation

Slow transforming viruses The standard oncogenic retroviruses, such as chronic leukemia viruses, are slow transforming viruses, that is they have low oncogenic potential and generally only induce malignant change of blood cells, after a long latent period. They do not transform cultured cells. They are capable of replicating normally.

Acute transforming viruses The acute transforming viruses are highly oncogenic and cause malignancy after a short latent period of weeks or months. They can cause different types of malignancies—sarcoma, carcinoma, leukemia—and also transform cells in culture. They can be:

- **Replication-defective** Most acute transforming viruses are unable to replicate normally because they carry on their genome an additional gene, the viral oncogene (***V-onc* gene**), which replaces some of the genes essential for viral replication. Such V-onc viruses can replicate only if co-infected with a standard helper retrovirus.
- **Replication-competent** The Rous sarcoma virus, which carries the oncogenic *src* (pronounced 'sark'), is the best known among the acute transforming viruses and can replicate normally because it possesses the full complement of *gag, pol* and *env* genomes.

ONCOGENES

Viral oncogenes (*V-onc*), commonly known as 'cancer genes', are genes that encode proteins triggering the transformation of normal cells into cancer cells
(Table 54.4). Oncogenes are not essential for the replication of the virus; mutants that lack these genes replicate normally without being oncogenic.

Table 54.4 Some oncogenes* and their chromosomal location in humans

Viral oncogene	Origin	Tumour/ malignancies	Human gene	Chromosomal location in human beings
V-src	Chicken	Sarcoma	*C-src*	20
V-ras	Rat	Sarcoma	*C-ras*	11
V-myc	Chicken	Leukemia	*C-myc*	8
V-fes	Cat	Sarcoma	*C-fes*	15
V-sis	Monkey	Sarcoma	*C-sis*	22
V-mos	Mouse	Sarcoma	*C-mos*	8

* Oncogenes are given three-letter codes from the animal or tumour from which they are derived, preceded by either V- or C-, for viral or cellular genes respectively; src = sarcoma of chicken, ras = rat sarcoma, sis = simian sarcoma, myc = myelomatosis of chicken, fes = feline sarcoma and mos = mouse sarcoma

Genes closely resembling viral oncogenes are found in normal as well as cancer cells. Oncogenes isolated from cancer cells are called **cellular oncogenes (*C-onc*)**. Similar genes found in normal cells are called **proto-oncogenes**. They are not of viral origin. On the contrary, viral oncogenes appear to be of host cell origin.

Cellular oncogenes contain introns characteristic of eukaryotic genes, whereas viral oncogenes do not. Apparently, viral oncogenes originated at some point in the distant past from the proto-oncogenes by recombination between retroviral and cellular genes.

Proto-oncogenes are widespread in vertebrates and metazoa—from human beings to fruitflies. They are well conserved in their genomes, suggesting that they serve some essential functions in normal cells. They have been found to code for proteins involved in regulating cell growth and differentiation. The presumed functions of many oncogenes have been identified. For example, the oncogene *src* is related to tyrosine-specific protein kinases, *sis* to a platelet-derived growth factor and *myc* to DNA-binding proteins, all concerned with the regulation of normal cell growth.

Transfection

This is a useful method for the study of oncogenes. Certain mouse fibroblast cell lines, such as NIH 3T3, can take up foreign DNA,

incorporate them into their genome and express them. This is called transfection. By this technique, DNA extracted from human tumour cells has been shown to transform 3T3 cells, and such transforming genes have been shown to be identical with cellular oncogenes.

Anti-oncogenes

A class of genes has been identified in the normal retinoblastoma (Rb) gene, the loss of which is associated with the development of retinoblastoma in children. The **p53 gene** appears to be a **tumour suppressor gene** with a wide range of effects. Specific chromosomal deletions, recognised in association with certain types of human cancers may reflect the loss of tumour suppressor genes.

Mechanisms of Viral Oncogenesis

While it is known that oncogenic viruses can transform cells in culture and induce tumours in animals under natural or experimental conditions, the exact mechanisms of viral oncogenesis are not well understood. Malignancy is a stable, heritable change and, as such, should be the result of a modification of the host cell genome.

Oncogenic DNA viruses

Here, the viral DNA (or a portion of it) is **integrated with the host cell** genome. As the viral DNA is incomplete or 'defective', no infectious virus is produced. However, under its influence, the host cell undergoes malignant transformation. A virus-transformed cancer cell is in many ways analogous to a bacterium lysogenised by a defective phage. In both cases, the cell is not destroyed and no virus is produced. Acquisition of new characteristics by the transformed cell resembles lysogenic conversion in bacteria.

Oncogenic RNA viruses

In general, retroviruses induce tumours by one of two mechanisms:

- They may introduce into the cellular genome a new transforming gene **(oncogene)**.

- **They may induce or alter the expression** of a pre-existing cellular gene. Several molecular mechanisms have been suggested for the conversion of benign proto-oncogenes to cancer genes. The genes may get overexpressed and the overproduced gene product may lead to abnormal growth. Recombination between retroviral and cellular genes, promoter insertion, chromosomal translocation, gene amplification and mutation are some of the genetic processes relevant in this connection.

MISCELLANEOUS VIRUSES

Human Papillomaviruses

Papillomaviruses are species-specific and infect squamous epithelial and mucous membranes, inducing different types of warts or papillomata in their hosts. The **human papillomavirus (HPV)** infects only humans and grows only in organ cultures of human skin. Over 82 types of HPV have been recognised based on genetic homology. There is a correlation between the virus type and the type of lesion produced.

Association of HPV with some clinical conditions

- Common warts (verruca vulgaris, plantar warts), usually found on the hands and feet of children and adolescents, are mostly caused by types 1, 2, 3 and 4.
- **Condyloma acuminatum** or genital wart, which is a more moist, soft, pedunculated wart found on the external genitalia, is usually due to types 6 and 11, 42–44. This may be transmitted venereally and may occasionally turn malignant.
- **Intraepithelial neoplasia** is due to HPV types **6 and 11**. There is a close association between specific HPV types and genital malignancies in both sexes.
- **Cervical cancer** is caused by HPV types **16 and 18**. Co-factors appear to be important in the induction of HPV-associated malignancies.
- **Laryngeal and oesophageal carcinomas** are associated with HPV types 16, 18, 30, 31, 33, 51–53.

HPV vaccine

Two recombinant vaccines are now available: one containing antigens from **HPV 6, 11, 16** and **18,** and a second, containing only particles from types **16** and **18**. Both are indicated in preventing persistent and precancerous genital lesions in adolescent and young adult women; they are

contraindicated in pregnancy.

Human Polyomaviruses

Human polyomaviruses (formerly, papovavirus group) have been isolated from a number of patients with impaired immunity. They contain the JC and BK viruses. Antibodies to these viruses are present widely in human sera, in about 75 per cent of adults. In an impaired immune system following disease or transplantation, the virus may be reactivated, leading to progressive multifocal leukoencephalopathy (PML) or renal disease.

The **JC virus** was isolated in 1971 from the brain of a patient with Hodgkin's disease and PML. This virus **grows only in human fetal glial cell cultures**. It is oncogenic, producing malignant gliomas following intracerebral inoculation in newborn hamsters.

The **BK virus** was isolated in 1971 from the urine of a patient who had undergone a kidney transplant. Several similar isolates have been reported from other kidney transplant patients. The BK virus can **grow in a wide range of primary and continuous cell cultures**.

Parvoviruses

Parvoviruses are small, 20 nm viruses with a single-stranded DNA genome. They are dependent on the host cell DNA for replication. There is only one medically important human parvovirus, **Parvovirus B19**. It was discovered in the blood of symptomless blood donors.

Parvovirus B19

It is present worldwide. Infection is commonly acquired in childhood and is often asymptomatic.

- ***Erythema infectiosum*** It usually presents as a respiratory infection with an erythematous maculopapular rash and arthralgia. It begins as a prominent erythema of the cheeks (slapped cheek disease), spreading to the trunk and limbs, followed by lymphadenopathy and arthralgia. It occurs usually in children who are 5–10 years old and has been called the fifth disease, as it was the fifth in the old list of six exanthematous fevers of children.

- ***Aplastic crisis*** It occurs in children with chronic hemolytic anemias, as in sickle cell disease. In the immunodeficient, it may cause persistent anemia.
- ***Fetal hydrops*** Parvovirus B19 infection during the second or third trimester of pregnancy leads to this condition.

Transmission appears to be respiratory, though it may also be through blood. Infection leads to viremia and virus replication in the throat, followed by antibody response. Diagnosis may be made by the detection of the virus in blood in early cases, and of the antibody later.

Bocavirus

A new parvovirus called bocavirus has been isolated from the respiratory tracts of children presenting with acute respiratory disease.

VIRAL HEMORRHAGIC FEVERS

Hemorrhagic manifestations are sometimes seen in viral fevers. However, the term hemorrhagic viral fever is not applied to all of them but only to a group of diseases, apparently **zoonotic** in nature, with features of hemorrhage caused by viruses belonging to two families: **Arenavirus** and **Filovirus**. Their distribution is geographically restricted to **South America** and **Africa**. They usually cause asymptomatic infection in the local population but at times they erupt into sudden outbreaks, killing large populations and causing panic.

Arenaviruses

A group of enveloped viruses with a negative-sense, single-stranded RNA genome, causing chronic inapparent infection in rodents has been classified as arenaviruses. Electron microscopy of thin sections shows characteristic electron-dense granules resembling grains of sand within the virus particles. The name of this group is derived from the Latin word *arena*, meaning sand. These particles are cellular ribosomes picked up by the virus, presumably during maturation by budding from host cells. Arenaviruses are spherical or pleomorphic particles, ranging in size from 80 to 300 nm.

Arenaviruses have assumed considerable medical importance after the recognition that some members of the family cause hemorrhagic fevers in

humans (Argentinian and Bolivian hemorrhagic fevers and Lassa fever).

South American hemorrhagic fevers

Two related viruses, the Junin and Machupo viruses, cause the Argentinian and Bolivian hemorrhagic fevers, respectively. They belong to the Tacaribe group of arenaviruses. Rodents act as reservoirs, and transmission is believed to occur through rodent excreta.

Lassa fever

This is the most highly publicised of viral hemorrhagic fevers and is caused by another arenavirus. It was first noticed in 1969 in an American Mission station in Lassa, Nigeria. Many outbreaks have subsequently occurred in widely separated foci in West Africa. The case fatality rate has been 35–70 per cent in hospitalised patients. The natural reservoir is the multimammate rat. Rodent excreta probably acts as the source of infection. The incubation period is 3–16 days. The virus is present in the throat, urine and blood of patients. Person-to-person transmission may occur by droplet infection. Nosocomial infection has occurred frequently. Ribavirin has proved useful in treatment.

Filoviruses

These are long, thread-like viruses, hence the name (*filum* meaning thread). They range in size from 80 to 800–1,000 nm. Ebolaviruses and Marburg viruses causing hemorrhagic fever belong to the genus *Filovirus*.

Ebola virus

In 1976, several cases of a similar hemorrhagic fever occurred in the equatorial provinces of Sudan and Zaire, with high fatality. The causative virus was morphologically identical to the Marburg virus but antigenically distinct. It has been called Ebola virus after the Ebola river, beside which the first cases were noticed (Fig. 54.2). In 1979, Ebola re-emerged in Sudan, with serial person-to-person spread. In 1995, a large outbreak with heavy fatality was seen in Kikwit, Zaire.

Fig. 54.2 Ebola virus (long, thin, filamentous form) (*Source:* Public Health Image Library, ID 23186 / Dr. Frederick Murphy / CDC)

Five distinct strains of Ebola virus have been recognised: the Ebola-Zaire strain (EBO-Z) with a case fatality rate of up to 90 per cent, the Ebola-Sudan strain (EBO-S) with a case fatality rate of up to 50 per cent, the mild Ebola-Reston strain (EBO-R) isolated from quarantined monkeys imported from the Philippines and held at Reston, Virginia, USA and Ebola-Ivory coast and Ebola-Bundybogio, which are found in Africa.

The recent outbreak between 2013 and 2016 in West Africa (Guinea, Liberia, Sierra Leone) resulted in more than 11,000 deaths.

The reservoir of Ebola virus and its natural history still remain unclear, despite frequent outbreaks with high fatality.

Pathogenesis High mortality is attributed to virulence factors like the glycoprotein which kills endothelial cells resulting in hemorrhage. It also kills the macrophages and lymphocytes. Viral proteins inhibit action of interferons.

It has a short incubation period of 5–7 days, which may go up to 21 days. Fever, headache, sore throat, myalgia, arthralgia, vomiting and diarrhea are some of the cardinal features. Subsequently, bleeding into the skin, marked thrombocytopenia and lymphopenia occur. Multiorgan failure leads to death. Mortality is 90%. A few may recover but the disease is followed by post-Ebola syndrome (PES) with blurring of vision, uveitis, arthralgia and fatigue. Ebola virus has been recovered from the fluid of the interior of the eyes several months after recovery.

Laboratory diagnosis RT-PCR for detecting the viral RNA or ELISA to detect the viral antigen in serum have been employed to confirm Ebola. Electron microscopy may also be used to demonstrate long, filamentous viruses. The virus can also be cultivated in monkey kidney cells (Vero and MA104). This is done in biosafety level 4 containment laboratories as the

patient's samples may contain viable virulent virus.

Treatment No antiviral treatment is available. Supportive therapy with stringent infection-control practices to prevent secondary spread by body fluids, with personal protective equipment is being followed. A monoclonal antibody (ZMapp) was tried with little success.

Vaccine Trials of a **vaccine rVSV-ZEBOV** have met with some success.

The current vaccination strategies for Ebola are as follows:

Innovative strategy

1. *Pop-up vaccination* To vaccinate all contacts in a protected vaccination site, i.e., health care facility, away from the residence of the contacts.
2. *Targeted geographic vaccination* To vaccinate all contacts and their contacts, in a particular geographic area; this is also done in a protected health care facility

A second revised vaccination strategy is to vaccinate all contacts and their contacts, including a third group of possible contacts at risk, to form a ring or barrier of immunised persons around the incident case.

Two other vaccines that are considered are:

i) Adenovirus 26 vectored glycoprotein/MVA-BN (Ad26.ZEBOV/ MVA-BN)

ii) Ad5-EBOV vaccine

[*WHO Ebola Virus Disease. Democratic Republic of the Congo External Situation Report 39. http://newsletters.afro.who.int/icfiles/1/ 46425/184054/ 6134450/97816cb57ede 15249d4eb5b5/ sitrep_evd_d rc_20190430-eng.pdf?ua*=1,]

Marburg virus

Marburg disease is a hemorrhagic fever that occurred simultaneously in laboratory workers in Marburg, Frankfurt (Germany) and Belgrade (Yugoslavia) in 1967. The infection arose from tissues of African green monkeys to which the laboratory workers had been exposed. The monkeys had been imported from Uganda. Person-to-person transmission occurred. The primary cases had a fatality rate of 30 per cent but the secondary cases were non-fatal.

The Marburg virus was isolated in guinea pigs and in tissue culture from

the blood and tissues of these patients. The virus appears to persist in the body and has been isolated after 80 days of onset of the illness from semen and the anterior chamber of the eye. A case of sexual transmission has also been recorded.

No further Marburg virus infections have been seen since except for three cases identified in South Africa in 1975 and two in Kenya in 1980.

Lymphocytic choriomeningitis (LCM)

The prototype is the **lymphocytic choriomeningitis (LCM)** virus which is a natural parasite of mice. Humans probably acquire the infection from the excreta of rodents. Most human infections are asymptomatic but some may develop an influenza-like illness or meningitis. LCM has been reported to account for 5–10 per cent of sporadic viral meningitis in human beings.

CORONAVIRUSES

A group of spherical or pleomorphic enveloped, single-sense RNA viruses, carrying petal- or club-shaped peplomers on their surface has been classified as coronaviruses. The name refers to the fringe of surface projections (spike proteins) surrounding the virus, resembling the solar corona
(Fig. 54.3). The spike proteins of the coronavirus help the virus to bind to the target cell membrane receptor.There are two groups of coronavirus: **acid-labile viruses** (associated with common cold-like illnesses) and **acid-stable viruses** (associated with human and animal gastroenteritis). Coronaviruses are further divided into four main subgroups—alpha, beta, gamma and delta—based on their genomic structure. Of these, only alpha and beta infect mammals. There are many serotypes, which are mostly fastidious and difficult to grow in cell culture systems.

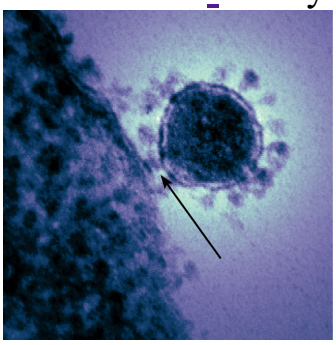


Fig. 54.3 Coronavirus: The spike protein of the virus binding to the cell membrane (*Source:*

Public Health Image Library,
ID 18114 / National Institute of Allergy and Infectious Diseases (NIAID) / CDC)

Coronavirus types

- Human coronavirus 229E
- Human coronavirus OC43
- SARS-CoV
- Human coronavirus NL63 (HCoV-NL63, New Haven coronavirus)
- Human coronavirus HKU1
- Middle East respiratory syndrome coronavirus (MERS-CoV), previously known as novel coronavirus 2012 and HCoV-EMC
- The strain implicated in the 2019–20 pandemic coronavirus infection was initially called the 'novel coronavirus' (NCoV) and the disease, SARSCoV2. It was subsequently named COVID-19 (coronavirus disease-19) as it first appeared in December 2019 in the Wuhan District of China.

Human coronaviruses were first isolated from cases of common cold by inoculating organ cultures of human embryonic trachea with nasopharyngeal washings. Inhibition of ciliary motility indicates virus growth. Inoculation in human volunteers induces the common cold after an incubation period of 2–5 days. The resulting immunity is poor and re-infections can occur even with the same serotype. They appear to be the second most common cause of the common cold, particularly in winter, next only to rhinoviruses.

Severe Acute Respiratory Syndrome (CoV-SARS)

In November 2002, Guangdong province in South China experienced an outbreak of an unusual respiratory infection with many deaths. The world outside knew about it only in February 2003, when a physician from Guangdong visited Hong Kong, fell ill and died there, after infecting 12 persons who had stayed in the same hotel. They, in turn, after returning to their countries, fell ill and initiated outbreaks there. By July, when the pandemic was controlled, it had affected over 30 countries, with many thousands of cases and over 800 deaths. India escaped the SARS epidemic; however, a few suspect cases were detected and quarantined.

The coronavirus responsible for SARS appears to be distinct from other coronaviruses, which had been classified into three types: CoV-SARS—**mammalian viruses in types 1 and 2** and **avian viruses in type 3**. The

present SARS virus is **coronavirus type 4**.

The fact that it is a new virus is indicated by the absence of an antibody to it in human and animal sera collected from previous years. It may be a recombinant of some animal and human viruses. The virus has been isolated from Chinese wild civets and raccoon dogs but not from pigs, dogs, cattle or poultry.

Spread SARS spreads by the inhalation of the virus present in droplets or aerosols of respiratory secretions of patients. Fecal aerosols also may be infectious. The incubation period is under 10 days. The disease starts as a fever with cough or other respiratory symptoms. Diarrhea is sometimes seen. The chest radiograph shows pneumonic changes. Death is due to respiratory failure.

Laboratory diagnosis The virus was identified by electron microscopy and confirmed by growth in Vero cell culture, animal inoculation, cloning, sequencing and histology. Molecular and serological tests for rapid diagnosis have been developed. Reverse transcription PCR has been used for early diagnosis, while demonstration of rise in titre of antibodies by ELISA or indirect immunofluorescent test in paired serum samples is useful later.

Treatment No specific therapy or prophylaxis has been identified. The virus is highly mutable and so vaccine prophylaxis may not be easy. Control has been achieved by strict isolation and quarantine.

MERS-CoV

Middle East respiratory syndrome (MERS) is a viral respiratory illness that has recently been reported. It was first detected in Saudi Arabia in 2012. The virus that causes MERS is called Middle East respiratory syndrome coronavirus (MERS-CoV). Coronaviruses are common viruses that infect most people at least some time in their lives. Human coronaviruses usually cause mild-to-moderate upper-respiratory tract illnesses. However, MERS-CoV is different from any other coronavirus previously found in people. This virus is thought to have come from animals including camels. Infection spreads through respiratory secretions. Another name for the virus is human coronavirus EMC (HCoV-EMC).

Symptoms Persons infected with MERS-CoV develop severe respiratory

illness. They have fever, cough and shortness of breath. Severe cases may have acute renal failure.

Treatment Currently, there is no vaccine to prevent the infection. Strict isolation and barrier nursing is the only way to prevent spread.

COVID-19

In December 2019, the first few cases of pneumonia with severe acute respiratory syndrome were admitted to a hospital in Wuhan in the Hubei province of China. The early COVID-19 cases were traced to the Huanan seafood market with a possibility of human-to-human transmissions. The disease spread rapidly within Wuhan and the neighbouring districts with high infectivity and mortality. From China, the disease spread to other parts of the globe, including Europe, the USA, the Middle East and parts of Asia including India, Africa and Australia. By May 2020, the WHO had declared it a pandemic. The total number of confirmed cases across the world on 4 May 2020 was 34,42,234 with 2,9,740 confirmed deaths. (Source: https://www.who.int/emergencies/diseases/novel-coronavirus-2019.) In India, the total number of recorded deaths due to COVID-19 as on 4 May 2020 was 1,568.

SARS-CoV2, the strain implicated in this pandemic, is a new β-coronavirus belonging to the subgenus of coronaviridae, closely related to the bat SARS-CoV. Its reservoir host is thought to be the pangolin, an ant-eating mammal. Soon after the outbreak, the genome of the virus was sequenced. It was found to share 79.5% of its genetic identity with the SARS-CoV that caused the 2002–2003 epidemic.

Pathogenesis Coronaviruses including SARS-CoV2 are covered with spike proteins that contain a variable receptor-binding domain (RBD). This RBD binds to the angiotensin-converting enzyme-2 (ACE-2) receptor found in the heart, lungs, kidneys and gastrointestinal tract, leading to damage of the alveolar and other cells. The disease starts with the antigen-presenting cells performing two functions: (i) presenting the foreign antigen to $CD4^+$ T helper (T_h1) cells and (ii) releasing interleukin-12 to further stimulate the T_h1 cell. The T_h1 cells stimulate CD8+ T killer (T_k) cells that target any cells that contain the foreign antigen. In addition, activated T_h1 cells stimulate B cells to produce antigen-specific antibodies.

The incubation period of SARS-CoV2 infection ranges from 2 to 14 days. Person-to-person transmission is efficient, with multiple clusters being reported. SARS-related damages to alveolar cells can, in turn, trigger a series of systemic reaction leading to a rise in body temperature > 38°C) with cough with dyspnea, accompanied by chest discomfort; patients also experience fatigue and muscle pain. A small number of patients also develop headaches, hemoptysis and diarrhea. Respiratory distress appears 7 days after the onset of symptoms and worsens within the next 2–3 days, requiring mechanical ventilation.

Epidemiology The pangolin is believed to be the intermediate host of SARS-CoV2. Infectivity of the virus is very high although case fatality is lower than that of MERS-CoV. Human-to-human transmission is the main mode of transmission. No seasonal predilection has been established although hot summer months are expected to bring down the incidence. As the pandemic unfolds, new information is being gathered regarding the epidemiology of the viral infection.

Laboratory diagnosis

1. *Imaging techniques* Abnormalities in computed tomography (CT) images of the chest are seen in almost 100% of the patients. Bilateral ground glass-like appearance and consolidation are also seen.
2. *Specimens* Recommended samples are oropharyngeal or nasopharyngeal swabs.
3. *Serological tests* IgG and IgM antibodies start to increase around 10 days after onset of symptoms with seroconversion within the first 3 weeks. Antibodies against the nucleoprotein and the surface spike receptor-binding domain correlate with neutralising activity.
4. *Molecular methods* The diagnosis is confirmed by detecting the viral RNA load by reverse transcriptase quantitative PCR (RT-qPCR).

Treatment Remdesivir, an adenosine analog, has been tried. Treatment has also been attempted with lopinavir and ritonavir, two antiretroviral drugs. Hydroxychloroquin is known to block virus–cell interaction and interfere with glycosylation of ACE2 and the CoVID-19 virus. The main mode of management is mainly symptomatic treatment and mechanical ventilation.

Prophylaxis The main strategy for prevention is social distancing and personal hygiene like hand washing and cough etiquette. The long-term

goal of SARS-COV2 research is to develop an effective vaccine that will yield neutralising antibodies against the viral antigen.

Key points

- Viruses that produce tumours in their natural hosts or in experimental animals, or which induce malignant transformation of cells on culture, are known as oncogenic viruses.
- Examples of oncogenic viruses in humans:
    - Epstein–Barr Virus (EBV), which causes Burkitt's lymphoma, common in Central Africa
    - Human papillomaviruses (HPV), associated with cervical cancer
    - HTLV-1 retrovirus, linked to leukemia
- Chronic infection due to the hepatitis B virus may lead to hepatocellular carcinoma.
- Papillomaviruses are species-specific and infect squamous epithelia and mucous membranes.
    - HPV types 1, 2, 3 and 4 cause common warts (verruca vulgaris).
    - HPV types 6 and 11 cause condyloma acuminatum (genital warts) and are also associated with intraepithelial neoplasia.
    - HPV 16 and 18 are causatively related to severe cervical cancer.
- Parvovirus B19 is clinically important, causing erythema infectiosum (also aplastic crisis in children with chronic hemolytic anemia).
- Hemorrhagic viral fevers are a group of zoonotic infections with typical hemorrhagic features, caused by viruses belonging to the Arenaviridae and Filoviridae families; the infections (usually asymptomatic) are localised to South America and Africa and may occur as sudden outbreaks.
- Coronaviridae is a family of markedly pleomorphic, enveloped, spherical RNA viruses that have helical nucleocapsids.
    - Severe acute respiratory syndrome (SARS) refers to a severe atypical pneumonia that assumed pandemic proportions in 2003. Since then, there has been no major outbreak.
    - A second coronavirus, MERS-CoV, was implicated in an outbreak that occurred in 2012. This virus strain is thought to have come from animals including camels.
    - A third outbreak in the form of a pandemic started in December 2019 and assumed enormous proportions by early 2020 with 512,701 cases across the globe and 23,495 deaths.

Questions

Short Answers

1. Oncogenic viruses of humans
2. Important papillomaviruses of medical importance and associated diseases

3. Viral hemorrhagic fevers
4. Describe the pathogenesis and epidemiology of the COVID-19 pandemic

CHAPTER 55

Antiviral Chemotherapy and Immunoprophylaxis

Chapter navigation

- Antiviral agents
- Viral vaccines

INTRODUCTION

While a large number of drugs are available to treat bacterial infections, antiviral agents to treat viral infections are limited in number, the reason being that viruses are obligate intracellular parasites, intimately associated with host cells. Antiviral agents must be capable of selectively inhibiting viral functions without damaging the host. Another limiting factor is the replication cycle of the virus which occurs during the incubation period, following which the virus spreads to the target cell before symptoms appear, limiting the accessibility of the drugs to the virus within the cells/organs. Yet another limiting factor is the latency of some viruses, which get incorporated into the host cell and cannot be reached by the drug. However, antivirals are needed to reduce the morbidity and mortality as well as economic loss caused by viral infections. There are increasing numbers of immunosuppressed patients who are at increased risk of infection and are in need of treatment. Despite all the limitations, there are several antiviral agents which target specific mechanisms during viral replication. These are now available for specific disease entities.

Historical perspective The first clinically useful antiviral drug was developed in 1960 when *N*-methylisatin-β-thiosemicarbazone (methisazone, marboran) was found to be effective against poxviruses. It was used successfully against eczema vaccinatum and for the prevention and treatment of smallpox. Shortly thereafter, smallpox was eradicated and the drug went out of use.

Targets for Drug Action

Molecular virology studies have identified virus-specific functions that can serve as targets for antiviral therapy (Table 55.1). Such targets include:

Table 55.1 Common antiviral agents and their sites of action

Action of the drug	Antiviral drug
Entry of the virus	• Amantadine
Inhibition of nucleic acid synthesis • Polymerase inhibition – Herpesvirus – HIV – HBV – HCV • Reverse transcriptase inhibition – HIV	• Acyclovir, ganciclovir, valacyclovir, penciclovir, cidofovir, foscarnet, vidarabine • Zidovudine, lamivudine, didanosine, abacavir • Adefovir, entecavir, tenofovir, telbivudine • Sofosbuvir, dasabuvir
	• Zalcitabine (ddC), Zidovudine (AZT)
Protease inhibitors • HIV • HCV	• Saquinavir, ritonavir, indinavir, nelfinavir, lopinavir • Telaprevir, boceprevir

- Attachment of virus to host cells
- Uncoating of the viral genome
- Viral nucleic acid synthesis
- Synthesis of viral proteins
- Assembly of the virus
- Release of virus particles

However, as mentioned earlier, these steps are intimately associated with host cells, hence the adverse effects of antiviral agents also affect the cells to some extent.

A number of compounds have been developed that are used in the treatment of some viral diseases.

Some of the infections that are treated with antiviral agents are:

- Herpesvirus infections

- HIV infections
- Influenza
- Hepatitis

Mechanisms of Action

These may vary among antivirals. Often, the drug needs to be activated by enzymes in the cell before it can act as an inhibitor of viral replication. Most selective drugs are activated by a virus-encoded enzyme in the infected cell.

Important targets for antiviral agents are drugs that act by:

1. **Inhibition of nucleic acid synthesis** by:
   a) viral nucleoside and nucleotide analogues that inhibit nucleic acid synthesis
   b) reverse transcriptase
2. **Inhibition of protein synthesis** by protease inhibitors.

Other mechanisms inlcude preventing viral entry into the cell and by interferon, which prevents viral replication.

INHIBITORS OF NUCLEIC ACID SYNTHESIS

Nucleoside and Nucleotide Analogues

The most common antiviral agents in use are **nucleoside analogues,** the mechanism of action of which is by inhibiting nucleic acid replication by inhibiting polymerases necessary for the replication. Some analogues get incorporated into the nucleic acid and block further synthesis or alter its function.

Analogues can inhibit cellular enzymes as well as virus-encoded enzymes. Virus variants resistant to the drug usually arise over time, sometimes quite rapidly. The use of combinations of antiviral drugs can delay the emergence of resistant variants. This can be done by combination therapy, as is done in the treatment of HIV.

Examples of nucleoside analogues are zidovudine [azidothymidine (AZT)], vidarabine (adenine arabinoside), acyclovir (acycloguanosine), lamivudine (3TC) and ribavirin.

Nucleotide analogues persist in cells for long, thereby increasing the

potency, e.g., Cidofovir. They differ from nucleoside analogues by an attached phosphate group.

Reverse Transcriptase Inhibitors

Nevirapine was the first member of the class of non-nucleoside reverse transcriptase inhibitors. It acts by binding directly to reverse transcriptase and disrupting the enzyme which is an essential part of RNA transcription. Resistant mutants emerge rapidly.

PROTEASE INHIBITORS

Saquinavir for the treatment of HIV was the first protease inhibitor to be approved. It inhibits the protease enzyme that is required for the replication of the virus in the latter half of its replicative cycle. Protease inhibitors cleave viral *gag* and *gag-pol* polypeptide precursors, which are required to form the mature virion core. Inhibition of the protease yields non-infectious virus particles. These drugs are ritonavir and indinavir.

OTHER TYPES OF ANTIVIRAL AGENTS

Unstinted research has led to the development of many antiviral agents, the need for which became urgent with the advent of the AIDS pandemic (Table 55.2).

Table 55.2 Antiviral agents

Class of drugs	Antiviral agents	Target	Antiviral spectrum
Nucleoside analogues	Acyclovir Trifluridine Vidarabine	Viral polymerase inhibition	VZV HSV
	Ganciclovir	Viral polymerase inhibition	CMV
	Lamivudine, Didanosine Stavudine	Reverse transcriptase inhibition	HIV-1, HIV-2, HBV
	Zidovudine (AZT)	Reverse transcriptase inhibition	HIV-1, HIV-2, HTLV-1
	Ribavirin	Blocks capping of viral mRNA	RSV, Influenza A and B

	Cidofovir	Viral polymerase inhibition	CMV, HSV
Protease inhibitors	Indinavir, Retonovir Saquinavir	HIV protease inhibition	HIV-1, HIV-2
RT inhibitor	Nevirapine	Reverse transcriptase inhibition	HIV-1
Neuraminidase inhibitor	Oseltamivir	Viral neuraminidase inhibition	Influenza A and B
Host cell penetration/ uncoating	Amantadine	Blocks viral uncoating	Influenza A
DNA polymerase inhibitor	Foscarnet	Viral polymerase inhibition	HSV, HBV, HIV-1
Integrase inhibitor	Raltegravir	HIV integrase inhibitor	HIV-1

- **Amantadine** (α-adamantanamine) is an agent that prevents the uncoating of the virus by blocking ion channel activity of the protein matrix (2 protein) prior to entry into the host cell. This is used to treat the influenza A virus, but not influenza B or C. It can be administered prophylactically to prevent the infection.
- **Fuzeon** is a large peptide that blocks the entry of HIV-1 into cells by preventing the cellular membrane fusion step during entry.
- **Foscarnet** (phosphonoformic acid) is an organic analogue of inorganic pyrophosphate. It selectively inhibits viral DNA polymerases and reverse transcriptases at the pyrophosphate binding site.

INTERFERONS

The IFNs are proteins that are coded by the host as members of the cytokine family and that inhibit viral replication. They are produced very quickly (within hours) in response to viral infection or other inducers and are one of the body's first responders in the defence against viral infection. IFNs play a pivotal role in the innate antiviral immune response. They also modulate humoral and cellular immunity and have broad cell growth regulatory

activities. Their antiviral effects are as follows:

- IFNs play a primary role in the non-specific defence of the host against viral infections. IFNs interface with viral infection in cultured cells. They are detectable soon after viral infection in animals, wherein the viral production is seen to decrease.
- IFN does not protect the virus-infected cell that produces it and IFN itself is not the antiviral agent. Rather, IFN moves to other cells, where it induces an antiviral agent.
- IFNs are almost always host species-specific in function but are not specific for a given virus. The replication of a wide variety of DNA and RNA viruses can be inhibited. When IFN is added to cells before infection, there is marked inhibition of viral replication but nearly normal cell function.
- IFNs are extremely potent. Very small amounts are required for function. It has been estimated that fewer than 50 molecules of IFN per cell are sufficient to induce the antiviral state.

Virus mechanisms to counteract interferon

Viruses display different mechanisms that block the inhibitory activities of IFNs on virus replication. Specific viral proteins function in the following ways.

- They block induction of expression of IFN (herpesvirus, hepatitis C virus, papillomavirus and rotavirus)
- They block the activation of the key protein kinase (adenovirus, herpesviruses)
- They may activate a cellular inhibitor of PKR (influenza, poliovirus)
- They block IFN-included signal transduction (adenoviruses, hepatitis B virus)
- They neutralise IFN-γ by acting as a soluble IFN receptor (myxoma virus)

RESISTANCE TO ANTIVIRAL AGENTS

Resistance to antiviral agents is an emerging problem. With a limited spectrum of drugs, resistance has posed serious problems.

One of the classic examples of such an event is the resistance of the HIV to antiretroviral therapy. This has necessitated the use of multiple drugs to overcome this challenge. Hence there is a first-line and a second line of antiretroviral therapy (ART). Influenza virus has also been shown to be resistant to antiviral therapy.

IMMUNOPROPHYLAXIS OF VIRAL DISEASES

Viral vaccines are used to confer protection in general, and are more effective than bacterial vaccines. Viral vaccines may be live or killed (Table 55.3).

Table 55.3 Viral vaccines in common use

Disease	Type of vaccine	Mode of preparation
Poliomyelitis	Live	Avirulent strains grown in monkey kidney cell culture
	Killed	Virulent strains grown in monkey kidney cell culture, formalin-killed
Rabies	Killed (Semple type)	Fixed virus grown in sheep brain and inactivated by phenol or beta-propiolactone
	Killed	Virus grown in cell culture and inactivated with beta-propiolactone
Yellow fever	Live (17D)	Attenuated virus grown in chick embryos and lyophilised
Japanese B encephalitis	Killed	Virus grown in mouse brain and inactivated by formalin
Varicella	Live	Attenuated virus grown in chick embryo fibroblast culture
Mumps	Live	Attenuated virus grown in human diploid cell culture
Influenza	Killed (subunit)	Virus disintegrated with sodium eoxycholate
	Live (attenuated)	Virus attenuated by serial passage in eggs
	Live (mutant)	*ts* mutants which are avirulent
	Live (recombinant)	Recombinants with surface antigens of new strains and growth characters of established strains
Measles	Live	Attenuated virus grown in tissue culture
Rubella	Live	Attenuated virus grown in tissue culture
Hepatitis B	Cloned subunit	HBsAg cloned in yeast
Hepatitis A	Killed	Formalin-inactivated tissue culture vaccine

Rotavirus	Live	Attenuated rotavirus
Kyasanur Forest disease	Killed	Formalin-inactivated tissue culture vaccine
HIV, Ebola, RSV and dengue		Vaccines under clinical trial or at the research level

Live vaccines are more effective than killed vaccines. The smallpox vaccine has been used as the sole tool for the global eradication of the disease. The early live vaccines were developed empirically from natural viruses (like Jenner's cowpox vaccine) or by attenuation by serial passage (like yellow fever vaccines). With the development of more precise genetic techniques, live vaccines have been developed by:

i) Plaque selection (Sabin vaccine for poliomyelitis)
ii) *ts* mutants (Influenza A vaccine)
iii) Recombination (Influenza)

Live vaccines have the following **advantages**:

- A single dose is usually sufficient to confer protection.
- They can be administered by the route of natural infection so that local immunity is induced.
- They induce immunity against the whole range of viral antigens.
- They induce cell-mediated immunity.
- They provide more effective and more lasting immunity than killed vaccines.

The live vaccines are more economical and are administered more conveniently, especially for mass immunisation. Some of them can be given as combined vaccines (e.g., measles–mumps–rubella vaccine).

They have the following **disadvantages**:

- **There is a remote risk of reversion to virulence.** The virus may spread from the vaccinees to immunodeficient or other high-risk contacts. (The positive aspect of this would be herd immunity, as in poliomyelitis.)

- Interference by pre-existing viruses may sometimes prevent a good immune response following live vaccination.

Live vaccines are heat-labile and have to be kept under refrigeration.

Killed vaccines have been prepared by **inactivating viruses** with heat, phenol, formalin or beta-propiolactone. (Ultraviolet irradiation is not satisfactory because of the risk of multiplicity reactivation.) Adverse reactions may also be reduced by the use of **'subunit vaccines**', in which the virus is split by detergents or other chemicals and only the relevant antigens incorporated in the vaccine. **Vaccine production by cloning the desired antigen in bacteria or yeast is becoming increasingly common, as has been done in hepatitis B.**

Killed vaccines have the **advantage** of:

- Stability and safety
- Being given in combination as polyvalent vaccines
- No danger of spread of the virus from the vaccinee

The **disadvantages** are that multiple injections are required and that local immunity and cell-mediated immunity are not induced.

Passive immunisation with human gamma globulin, convalescent serum or specific immune globulin gives temporary protection against many viral diseases such as measles, mumps and hepatitis. These are indicated only when immunocompromised individuals are exposed to infection.

Combined active and passive immunisation is an established method for the prevention of rabies.

In the case of needlestick injury or exposure to blood products of hepatitis B-positive individuals, a combination of passive and active immunisation is done for individuals who have not been vaccinated earlier.

Key points

- Specific functions that serve as targets for antiviral therapy are:
  - Attachment of virus to host cells
  - Uncoating of the viral genome
  - Viral nucleic acid synthesis
  - Synthesis of viral proteins

    - Assembly of the virus
    - Release of virus particles
- Important targets for antiviral agents are drugs that act by:
    - **Inhibition of nucleic acid synthesis** by (a) viral nucleoside and nucleotide analogues that inhibit nucleic acid synthesis and (b) reverse transcriptase
    - **Inhibition of protein synthesis** by protease inhibitors
- Other mechanisms include preventing viral entry into the cell and by interferon which prevents viral replication.
- Interferons are proteins that are coded by the host as members of the cytokine family and that inhibit viral replication. They are produced very quickly (within hours) in response to viral infection or other inducers and are one of the body's first responders in the defence against viral infection.
- Some of the infections that are treated with antiviral agents are:
    - Herpesvirus infections
    - HIV infections
    - Influenza
    - Hepatitis
- Vaccines have proved to be effective in reducing the incidence of viral disease. Vaccination is the most cost-effective method of prevention of serious viral infections.
    - Killed virus vaccines—Inactivated (killed virus) vaccines are made by purifying viral preparations to a certain extent and inactivating by mild formalin in a way that it does not damage viral structural proteins.
    - Attenuated live virus vaccines—These are virus mutants that antigenically overlap with the wild-type virus but do not have the same pathogenic effects. Attenuated live virus vaccines have the advantage of acting like the natural infection.

Questions

Essay

1. Describe the mechanism of action of the major antiviral drugs. How do viruses develop resistance to some of these drugs?

Short Answers

1. Common viral infections that can be treated with antiviral drugs
2. Antiviral action of interferons
3. Advantages of live attenuated viral vaccines

Part V:
Medical Mycology

Part V: Medical Mycology

Chapter 56

Basic Mycology and Superficial Mycoses

Chapter navigation

- Introduction
- Classification of fungal diseases
- Superficial mycoses
  - Cutaneous mycoses
  - Subcutaneous mycoses

INTRODUCTION

Mycology (from the Greek word *mykos*, meaning fungus) is the branch of microbiology that deals with the study of fungi (yeasts and moulds). Medical mycology deals with the etiology, pathogenesis, detection, prevention and treatment of fungal infections in humans.

Fungi were recognised earlier than bacteria as the causative agents of human disease. Fungi that cause favus (*Trichophyton schoenleinii*) and thrush (*Candida albicans*) were described as early as 1839. Unfortunately, despite these early beginnings, the study of pathogenic fungi received only scant attention in comparison with the study of other microbial pathogens. This is probably due to the relatively benign nature of the common mycotic (fungal) diseases, their slow-growing nature and the absence of diagnostic tests other than morphological identification. There are millions of different fungal species on earth but only about 300 of these are pathogenic to humans.

Fungal infections are gaining importance due to their ubiquitous nature, some of them leading to serious infections, even fatal at times. Increased life expectancy due to treatment with chemotherapeutic agents, steroids,

immunosuppressive agents and antibiotics in moribund patients has led to an increase in opportunistic fungal infections. In the USA, fungal infections reportedly cause more fatalities than whooping cough, diphtheria, scarlet fever, typhoid, dysentery and malaria put together. Most fungi are saprophytes in the soil and human mycotic infections are mainly opportunistic, barring a few true pathogens.

CHARACTERISTICS OF FUNGI

Fungi are eukaryotic protista that differ from bacteria and other prokaryotes. Some of their different characteristics are as follows:

- They possess rigid cell walls containing chitin, mannan and other polysaccharides.
- The cytoplasmic membrane contains sterols.
- They possess true nuclei with nuclear membrane and paired chromosomes.
- They divide asexually or sexually, or by both processes.
- They may be unicellular or multicellular.
- The cells show various degrees of specialisation.

CLASSIFICATION OF FUNGI

Like most other microorganisms, fungi also possess different characteristics and may be classified based on the following:

I. Morphological forms
II. Type of infection produced by the fungi
III. Site of infection
IV. Type of sporulation

I. Classification Based on Morphological Forms

From a diagnostic point of view, fungi may be classified depending on cell morphology into four groups as given below (Fig. 56.1 and Fig. 56.2).

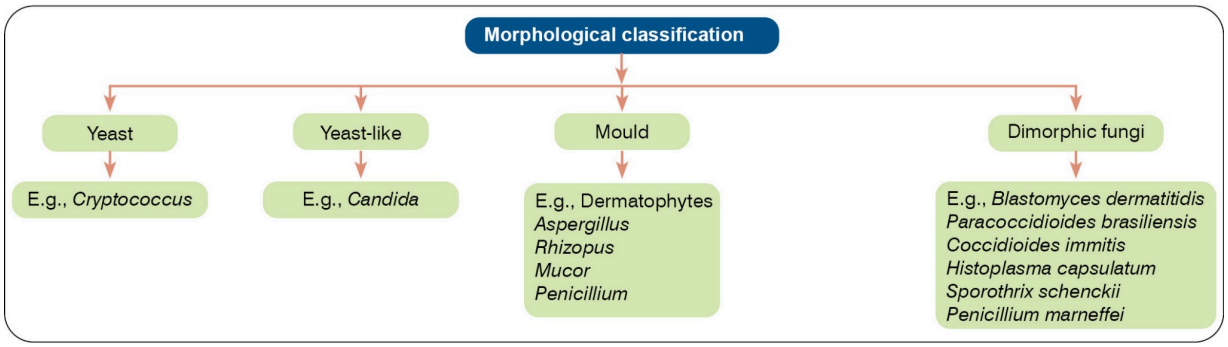


Fig. 56.1 Morphological classification of medically important fungi

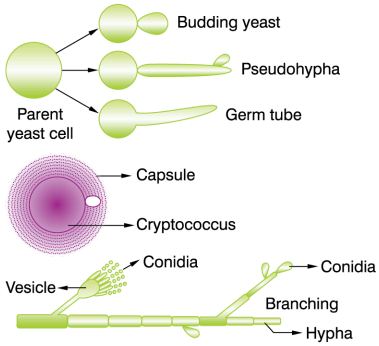


Fig. 56.2 Morphological forms of fungi: budding yeast and branching mould

1. Yeasts (e.g., *Cryptococcus neoformans*)

- They are unicellular fungi that do not produce germ tubes or pseudo-hyphae.
- They reproduce by budding (known as **blastospore** or **blastoconidium**).
- Macroscopic appearance of the colony is pasty, resembling bacterial colonies in culture.
- Under the microscope, the yeast cell appears as oval to round (3–15 ∝m in diameter) forms in tissues and in culture.
- Filamentous (hyphae-like) structures not seen in tissues or in culture (Fig. 56.3a and b).

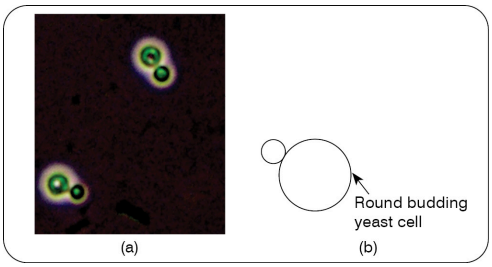


Fig. 56.3 (a) Budding yeast cell: *Cryptococcus neoformans* in India ink preparation and (b) round, budding yeast cell (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

2. Yeast-like fungi (e.g., *Candida albicans*)

- They are unicellular fungi.
- They reproduce by budding and by fission.
- Macroscopically, colonies appear pasty in culture like those of other yeasts (resembling bacterial colonies).
- Microscopic appearance of the cells are spherical or oval forms in tissues and in culture; filamentous structures (**pseudohyphae**) may be seen in clinical material (Fig. 56.4a, b and c).

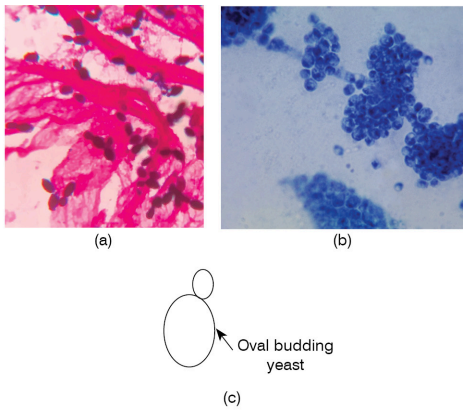


Fig. 56.4 (a) Oval budding cells of *Candida albicans* in Gram smear; (b) pseudohyphae and (c) oval budding yeast cells (*Source:* (a) Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry and (b) Public Health Image Library, ID 2918 / Dr. Gordon Roberstad / CDC).

3. Filamentous fungi or moulds (e.g., *Aspergillus, Penicillium* and dermatophytes)

- They are made up of hyphae which may have cross-walls or septa (multicellular) or may be devoid of septa (coenocytic), with conidiophores bearing fruiting bodies and asexual spores.
- They reproduce by asexual means (spore formation); some exhibit sexual reproduction.
- Macroscopic appearance of colonies: surface texture may be cottony/woolly/velvety/granular; pigmentation may be observed from the reverse.
- Microscopic appearance—thread-like filamentous hyphae (2–10 μm) seen in tissues and in culture. These may be **aseptate** (without cross-walls, e.g., *Zygomycetes*) or **septate** (with cross-walls, e.g., *Aspergillus fumigatus)* (Fig. 56.5a). Mycelium may be **vegetative** or **aerial**. Hyphae may take any of the following shapes: racquet, nodular, pectinate, spiral, root-like (rhizoid) or chandelier-like (a repeatedly branched cluster of hyphal apices that resembles a chandelier) (Fig. 56.5b).

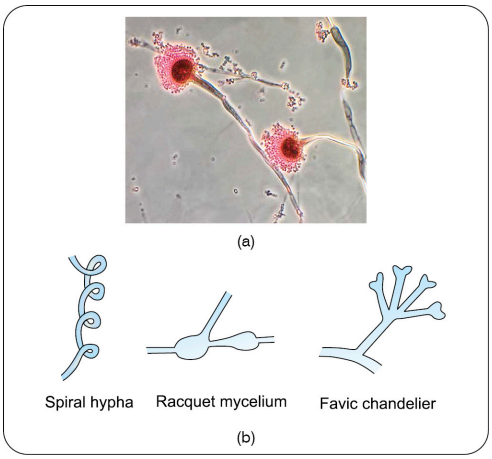


Fig. 56.5 (a) Aspergillus and (b) different forms of hyphae (*Source:* (a) Public Health Image Library, ID 15145 / CDC)

4. Dimorphic fungi (e.g., *Histoplasma capsulatum*)

- Grow as filamentous forms in culture at 22–25°C (Fig. 56.6a) and in the natural environment.

- Grow as yeast forms in culture at 37°C and in tissues (Fig. 56.6b).

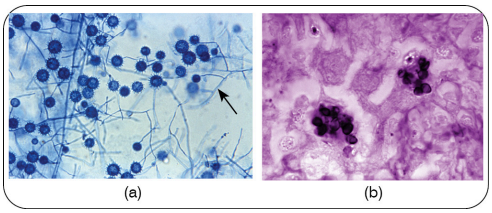


Fig. 56.6 *Histoplasma capsulatum*: (a) Mycelial (filamentous) form and (b) yeast form in tissue section (*Source:* (a) Public Health Image Library, ID 19420 / Dr. Lucille K. Georg /CDC and (b) Public Health Image Library, ID 19420 / Dr. Libero Ajello / CDC)

II. Classification Based on Type of Infection Caused by the Fungi

This depends on the pathogenic potential of the fungus and the immune status of the host.

Primarily pathogenic

Fungi that cause infections in healthy individuals, superficial mycoses, cutaneous mycoses, subcutaneous mycoses and deep or systemic fungal infections by dimorphic fungi, e.g., *Coccidioides immitis* (Fig. 56.7), fall into this category.

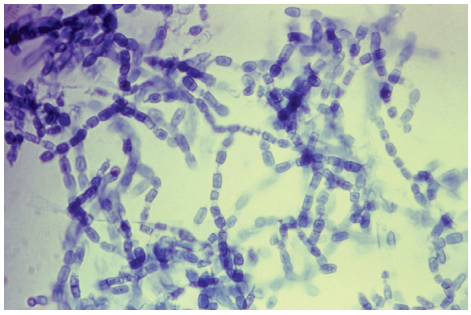


Fig. 56.7 Chains of arthrospores of *Coccidioides immitis,* which arise from the mycelia (*Source:* Public Health Image Library, ID 16303 / Dr. Brinkman / CDC)

Opportunistic pathogens

Fungi that cause infections by various mechanisms in patients who are immunocompromised fall into this category (e.g., individuals receiving immunosuppressive drugs, individuals infected with HIV, individuals who have diabetes mellitus or other debilitating conditions like aspergillosis,

zygomycoses, cryptococcosis).

III. Classification Based on Site of Infection

Fungal infections in humans are broadly classified based on their infection of tissues at various sites of the body (Table 56.1).

a. Superficial (dealt with in this chapter)

- Cutaneous mycoses
- Subcutaneous mycoses

b. Deep (systemic) (dealt with in Chapter 57)

- Deep mycoses (primary systemic infections of pathogenic fungi)
- Opportunistic mycoses (there is actual invasion of the tissue by the saprophytic fungi)

Table 56.1 Major mycoses and the diseases

Category	Fungal diseases
Superficial (localised to the stratum corneum)	Pityriasis versicolor, tinea nigra, white piedra, black piedra (produce concretions in the hair due to fungal growth)
Cutaneous	Dermatophytosis, candidosis of skin, nail and mucosa
Subcutaneous	Mycetoma, chromomycosis sporotrichosis, phaeohyphomycosis
Deep or systemic (often endemic)	Histoplasmosis, coccidioidomycosis blastomycosis, paracoccidioidomycosis
Opportunistic	Candidosis, cryptococcosis, aspergillosis, penicilliosis, zygomycosis, pneumocystis pneumonia

IV. Classification Based on Sporulation

Based on their method of sporulation, fungi are classified as follows:

Phycomycetes

These are the lower fungi (comprising the simplest and most primitive group of true fungi) that have **non-septate hyphae** and form endogenous, asexual spores called sporangiospores, contained within swollen sac-like structures called **sporangia**. They also produce sexual spores known as **oospores** in some fungi and **zygospores** in others.

The other three classes given below are the higher fungi that have septate hyphae and form exogenous asexual spores called **conidia**.

Ascomycetes

These form sexual spores (**ascospores**) within a sac or ascus. They include both yeasts and filamentous fungi.

Basidiomycetes

They form sexual spores (**basidiospores**) on a 'basidium' or base.

Deuteromycetes

These are **fungi imperfecti**; they are also called hyphomycetes, which is a group consisting of fungi whose sexual phases have not been identified. **Most fungi of medical importance belong to this group.**

Hypersensitivity (allergic reactions)—dermatophytid or Id reaction
Dermatophytid is an allergic rash caused by an inflammatory fungal infection (dermatophytic or tinea infection) at a distant site. Like dermatitis, the rash is often itchy, with blisters scattered on face, trunk and/or limbs. The fungus cannot be cultured from the allergic site. The condition can be treated with topical steroids. It generally resolves once the original infection has been controlled.

SUPERFICIAL MYCOSES

Superficial mycoses is a group of infections caused by fungi that live exclusively on the dead layers of the skin and its appendages. They have no contact with living tissue and, hence elicit no inflammatory response; the only changes produced are cosmetic effects. Examples:

i. Tinea (pityriasis) versicolor
ii. Tinea nigra
iii. Piedra

I. Cutaneous Mycoses

1. Pityriasis Versicolor (Tinea Versicolor)

This is a chronic, usually asymptomatic, involvement of the stratum corneum.

Distribution

The disease is worldwide in distribution but is particularly prevalent in the tropics. It occurs mainly in young adults.

Causative agent

The lipophilic, yeast-like fungus, *Malassezia furfur* (formerly, *Pityrosporum orbiculare*), is the causative agent.

Clinical features

Characteristic discrete or confluent macular areas of discolouration or depigmentation develop on the skin of the chest, abdomen, upper limbs and back. The fungus may be demonstrated on normal skin. They have been found in human dandruff and seborrheic dermatitis. The skin of most, if not all, warm-blooded animals is covered with *Malassezia furfur*, and while they mostly co-exist with their hosts, they can cause serious diseases in humans and other animals; the disease may also be considered an opportunistic infection (Fig. 56.8a and b).

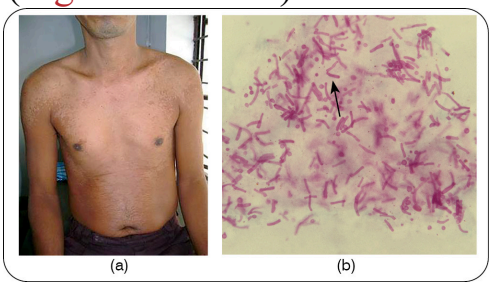


Fig. 56.8 (a) Pityriasis versicolor: macular area of discolouration on the upper chest wall and (b) photomicrograph of a skin scale tissue sample showing presence of numerous *Malassezia furfur* yeast and short filamentous forms (spaghetti and meatball appearance) (*Source:* (a) Dr Sheela Kuruvila, Department of Dermatology, Pondicherry Institute of Medical Sciences, Puducherry and (b) Public Health Image Library, ID 2916 / Dr. Lucille K. Georg / CDC)

Laboratory diagnosis

Examination of skin scrapings shows an abundance of yeast-like cells and short, branched filaments (spaghetti and meatball appearance) (Fig. 56.8b). The fungus can be grown on Sabouraud agar, covered with a layer of olive oil at 30–35°C. The colonies appear pale yellowish-brown to cream-

coloured in 5–6 days.

2. Tinea Nigra

This is a localised infection of the stratum corneum, particularly of the palms, producing black or brownish macular lesions.

Distribution

This disease mainly occurs in the tropics.

Causative agent

Exophiala werneckii (formerly, *Cladosporium wernickii*, *Hortaea wernickii*) and *Exophiala castellanii* are the organisms responsible.

Laboratory diagnosis

Skin scrapings show brownish, branched, septate hyphae and budding cells. Colonies on Sabouraud agar are grey or black.

3. Piedra

This fungal infection of the hair is characterised by the presence of firm, irregular nodules along the hair shaft; these nodules are composed of fungal elements cemented together on the hair. Two varieties of piedra are recognised: **black piedra** (Fig. 56.9) caused by *Piedraia hortae* and **white piedra** caused by *Trichosporon beigelii.*

Another group of fungi that cause cutaneous infections are the dermatophytes; the infection is known as dermatophytosis.

Fig. 56.9 Photomicrograph reveals pathologic morphology displayed by a hair shaft in black piedra, also known as trichosporosis, caused by *Piedraia hortae* (*Source:* Public Health Image Library, ID 12532 / Dr. Lucille K. Georg / CDC)

Dermatophytosis

The most important cutaneous infection is **dermatophytosis**, which is caused by a group of related fungi called the **dermatophytes**. Infection is

generally confined to the cornified layer of the skin and its appendages. Various inflammatory and allergic responses are induced in the host by the presence of the fungi and their metabolic products. *Candida albicans* may also cause infections of the skin and mucosa as well as systemic disease. *Candida* infection, therefore, represents a bridge connecting superficial and deep mycoses.

Dermatophytosis (commonly called tinea or ringworm) refers to infection of keratinised structures (the skin, hair and nails) caused by a group of keratinophilic fungi called the **dermatophytes**. The infections caused may be acute or chronic (persistent dermatophytosis that runs a chronic course with episodes of remission and exacerbation). The term dermatophytosis should not be confused with **dermatomycosis,** which refers to skin lesions produced by other fungi such as *Candida albicans* and also the cutaneous manifestations of systemic mycoses.

Characteristics of dermatophytes

Dermatophytic fungi are hyaline filamentous fungi that digest keratin by enzymatic means but are unable to invade living tissue. Dermatophytes digest keratin by keratinases and are resistant to cycloheximide. They are classified into three genera:

i) Microsporum ii) Trichophyton iii) Epidermophyton

Microsporum contains several species (e.g., *M. gypseum*, *M. canis* and *M. nanum*), as does *Trichophyton* (e.g., *T. rubrum*, *T. mentagrophytes* and *T. verrucosum*). *E. floccosum* is the only species in the genus *Epidermophyton*. About 40 species of dermatophytes are known to cause infections in humans and animals.

Clinical types of dermatophytosis

Dermatophytosis is twice as common in males as in females. Clinical classification is according to the anatomic site involved:

- Tinea barbae (barber's itch) involves the bearded areas of the face and neck.
- Tinea corporis (tinea glabrosa) is ringworm of the smooth or non-hairy skin of the body (Fig. 56.10a and b).

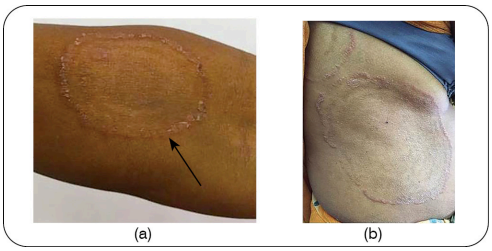


Fig. 56.10 (a) and (b) Tinea corporis: circumscribed lesions on non-hairy parts of the body (*Source:* Dr Sheela Kuruvila, Department of Dermatology, Pondicherry Institute of Medical Sciences, Puducherry)

- Tinea imbricata is a special type of tinea corporis found in the tropics, which presents with characteristic extensive concentric rings of papulosquamous, scaly patches.
- Tinea capitis is ringworm of the scalp; favus and kerion are variants.
- Tinea cruris (jock itch) involves the groin and perineum.
- Tinea pedis (athlete's foot) is ringworm of the foot.
- Tinea manuum involves the hand.
- Tinea unguium involves the nails.

Table 56.2 lists the clinical types of dermatophytoses and their common causative agents.

Table 56.2 Clinical types of dermatophytoses and their common causative agents

Disease	Common causative agents
Tinea capitis	*Microsporum* any species *Trichophyton* most species
Favus	*T. schoenleinii*, *T. violaceum, M. gypseum*
Tinea barbae	*T. rubrum, T. mentagrophytes,* *T. verrucosum*
Tinea imbricata	*T. concentricum*
Tinea corporis	*T. rubrum* and any other dermatophyte
T. cruris	*E. floccosum, T. rubrum*
T. pedis	*T. rubrum, E. floccosum*
Ectothrix hair infection	*Microsporum* species, *T. rubrum,* *T. mentagrophytes*

Endothrix hair infection	*T. schoenleinii, T. tonsurans, T. violaceum*

Clinical features

Lesions in the skin tend to be circular, dry, erythematous, scaly and itchy. Lesions of the hair include kerion, scarring and alopecia.

- **Favus** A chronic type of ringworm in which dense crusts (scutula) develop in the hair follicles, leading to alopecia and scarring.
- **Kerion** These are severe boggy lesions with marked inflammatory reaction that sometimes develop in the scalp infection due to dermatophytes (Fig. 56.11).
- **Nails** (Tinea unguium) Finger and toenails infected by dermatophytes are deformed, friable and discoloured, and there is accumulation of debris under the nails. In lesions, dermatophytes appear as hyphae and arthrospores.

Fig. 56.11 Kerion: boggy lesions on the scalp (*Source:* Dr Sheela Kuruvila, Department of Dermatology, Pondicherry Institute of Medical Sciences, Puducherry)

Pathogenicity

Mechanisms of pathogenesis are unclear. Fungal products may be responsible for inciting local inflammation. Hypersensitivity to fungal antigens results in sterile vesicular lesions sometimes seen in sites distant from the ringworm. The reaction may follow oral antifungal therapy and can be confused with an allergic drug reaction. These lesions are called **dermatophytids** (or the 'Id' reaction).

Diagnosis

Diagnosis is established by clinical features, the use of a Wood's lamp where applicable and by laboratory investigations.

Wood's lamp examination for detecting superficial fungal infections
This test is performed in a dark room by shining an ultraviolet light on the area of interest. A Wood's lamp allows only the long ultraviolet rays to pass (peak at 365 nm) through a nickel oxide filter (barium silicate with nickel oxide). Wood's lamp examination is useful for the diagnosis of certain ectothrix infections of the skin or scalp. Normally, the skin does not fluoresce or shine under ultraviolet light. However, if the region of the skin on which the Wood's lamp light is focussed is infected, that area of the skin will fluoresce. In particular, if the skin or hair is infected by the ***Microsporum*** species, (e.g., *M. canis, M. audouinii*), the infected area will fluoresce a bright greenish-yellow. **Malassezia** infections (*Pityriasis versicolor*) show golden-yellow fluorescence. A Wood's lamp reveals pteridine fluorescence of the infected hair.

Laboratory investigations

1. **Specimens** Scrapings of the skin (from the edges of ringworm lesions) and nail and hair clippings (hair plucked from the scalp) are collected for investigations.

2. **Microscopic examination** A wet preparation of the specimen is made by placing the scrapings in a drop of 10–20% potassium hydroxide (KOH) on a slide, which is then covered with a coverslip and left for 10–20 minutes to digest the keratin. Additional time may be required to digest nails. Digestion of keratin ('clearing') is aided by gently warming the slide. A positive finding by direct microscopic examination of the specimen establishes the diagnosis of ringworm, irrespective of whether culture is performed. The presence of **branching hyaline (non-pigmented) septate hyphae** is considered positive for fungi; spores may sometimes be seen (Fig. 56.12).

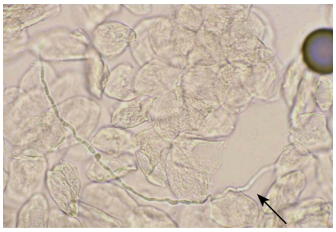


Fig. 56.12 Dermatophyte hyphae in skin scraping, KOH mount

In suspected tinea capitis, fungal elements are looked for in plucked hair. Selection of infected hair for examination is facilitated by exposure to UV light **(Wood's lamp**). Infected hair will be fluorescent. Two types of hair infection may be distinguished in wet mounts
(Fig. 56.13a): **ectothrix**, in which arthrospores are seen as a sheath surrounding the hair, and **endothrix**, in which the spores are inside the hair

shaft.

Hair perforation test This test is done to differentiate *Trichophyton rubrum* from *Trichophyton mentagrophytes*. A few strands of human hair are placed in a petri dish with 20 mL of distilled water, and autoclaved. A few drops of sterile 10% yeast extract and a few fragments of test fungus are added to the hair strands. These are incubated at 25°C for 2–3 weeks. The hair is then removed and examined microscopically in a lactophenol cotton blue-stained wet preparation. *T. mentagrophytes* causes surface erosion of the hair shaft, resulting in a wedge-shaped appearance of the shaft (Fig. 56.13b and c).

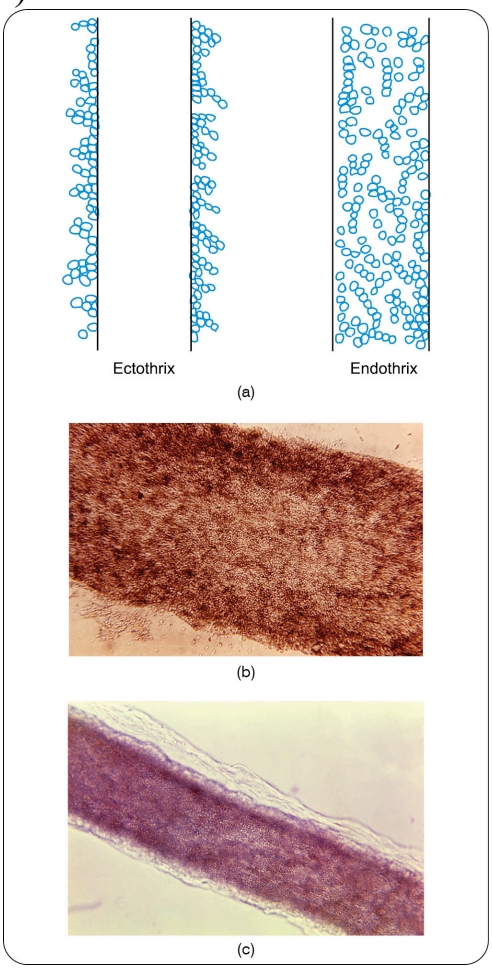


Fig. 56.13 (a) Ectothrix and endothrix types of hair infection and (b and c) photomicrographs of ectothrix and endothrix respectively (*Source:* (b) Public Health Image Library, ID 16625 / CDC / Dr. Lucille K. Georg / CDC and (c) Public Health Image Library, ID 22026 / CDC/ Dr. Lucille K. Georg / CDC)

3. **Culture** Species identification is possible only by examination of the culture. The specimen is inoculated onto:

- Sabouraud dextrose neopeptone agar containing chloramphenicol (the antibiotic suppresses the growth of the contaminating bacteria)
- Sabouraud agar containing chloramphenicol and cycloheximide (to suppress bacteria and non-dermatophytic filamentous fungi)

The plates are incubated aerobically at 25–30°C for up to 21 days and are checked daily for the appearance of fungal colonies. Identification of dermatophytes in the laboratory is by examining the **macroscopic characteristics** of the fungal colonies (rate of growth, texture, colour on the obverse and reverse) (Table 56.3).

Table 56.3 Common characteristics of dermatophytes

Species	Colony	Morphology
T. rubrum	Velvety, red pigment on reverse	Few, long, pencil-shaped macroconidia
T. mentagrophytes	White to tan, cottony or powdery Pigment variable	Clusters of microconidia Cigar-shaped macroconidia with terminal rat-tail filaments
T. tonsurans	Cream or yellow, with central furrows	Abundant microconidia Thick-walled, irregular macroconidia
T. schoenleinii	Smooth, waxy, brownish	Hyphal swellings, chlamydospores, favic chandelier
T. violaceum	Very slow-growing, waxy, violet/purple pigment	Distorted hyphae, conidia rare
M. audouinii	Velvety, brownish, slow-growing	Thick-walled chlamydospores, conidia rare and irregular
M. canis	Cottony, orange pigment on reverse	Abundant, thick-walled, spindle-shaped macroconidia with up to 15 septa
M. gypseum	Powdery, buff-coloured	Abundant, thin-walled macroconidia with 4–6 septa
E. floccosum	Yellowish green, powdery	Club-shaped macroconidia in clusters

- ***Trichophyton*** Colonies may be powdery, velvety or waxy, with pigmentation characteristic of different species.

- ***Microsporum*** Colonies are cotton-like, velvety or powdery, with white to brown pigmentation.
- ***Epidermophyton*** Colonies are powdery and greenish yellow.

Microscopic examination of material teased out from the fungal colony (culture mount) or material taken on cellotape (cellotape mount) or slide culture is done. Growth is carefully transferred to a slide so that the structural arrangement is preserved and suspended in a drop of alcohol. A wet preparation is made by staining the growth with lactophenol cotton blue. The hyphae and conidia are observed. Conidia (asexual spores) are of two types: small, unicellular microconidia and larger, septate macroconidia.

- ***Trichophyton*** Microconidia are abundant and arranged in clusters along the hyphae or borne on conidiophores (Fig. 56.14). Macroconidia are relatively scanty, generally thin, elongated, with blunt ends and have distinctive shapes in different species, which aids species identification. Some species possess special hyphal characters, such as spiral hyphae, racquet mycelium and favic chandeliers. Trichophyton species infect skin, hair and nails.

  T. rubrum (*Clinical case* 1), the most common species infecting human beings, often causes chronic, treatment-resistant lesions.

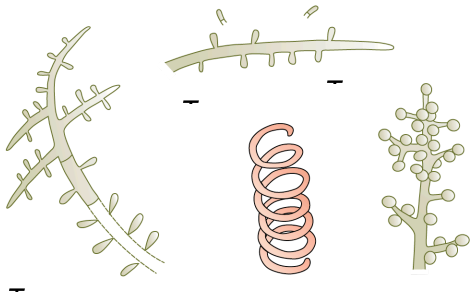


Fig. 56.14 *Trichophyton* species showing typical spiral hyphae and microconidia

- ***Microsporum*** Microconidia are relatively scanty and not distinctive. Macroconidia, the predominant spore form, are large, multicellular, spindle-shaped structures, borne singly on the ends

of hyphae. *Microsporum* species infect the hair and skin but usually not the nails.

- ***Epidermophyton*** Colonies are powdery and greenish-yellow. Microconidia are absent. Macroconidia are multicellular, pear-shaped and typically arranged in clusters (Fig. 56.15). *Epidermophyton* attacks the skin and nails but not the hair.

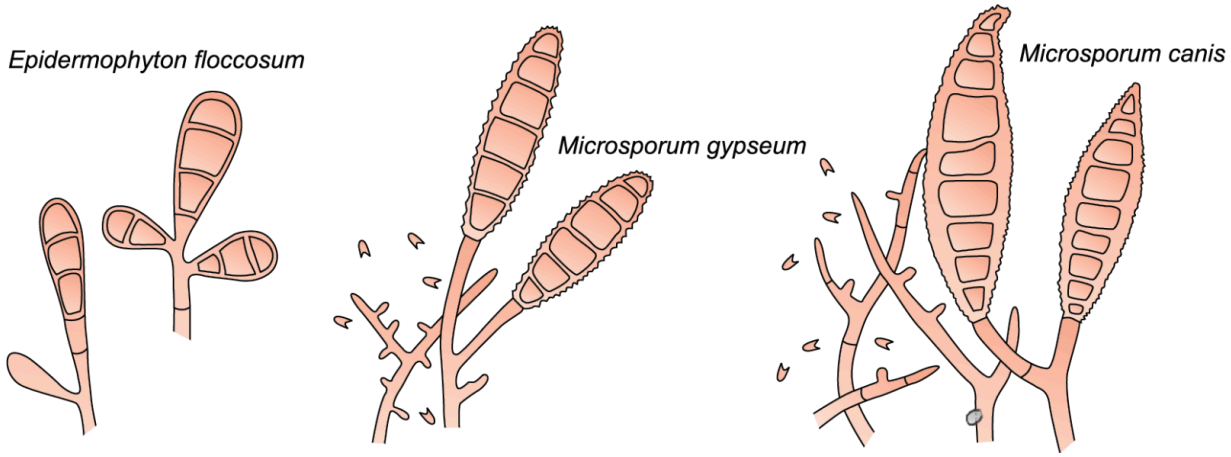


Fig. 56.15 Characteristic macroconidia

Clinical Case 1

A nine-year-old boy presented at the Dermatology OPD complaining of change in the colour of his toenails for about four months. He had no history of trauma or eczema of the foot. On examination, onycholysis (separation of the nail plate from the nail bed) of multiple toenails was observed. The nails were clipped and a 20% potassium hydroxide preparation on microscopy revealed the presence of thin, septate hyphae. Culture on Sabouraud dextrose agar containing cycloheximide showed the growth of mycelial fungi, which on lactophenol cotton blue (LCB) mount, revealed the presence of slender, septate hyphae with a few pencil-shaped macroconidia and many oval microconidia. This was identified as Trichophyton rubrum (the most common pathogen in onychomycosis). The patient was treated with oral griseofulvin and recovered.

4. **Physiological tests** Identification of species is based on certain physiological tests, such as testing the ability of the fungus to penetrate the hair under experimental conditions (in vitro hair perforation test), their ability to hydrolyse urea, grow on polished rice grains and on certain special media and withstand elevated temperatures (temperature tolerance and enhancement). The requirement of the fungus for certain special amino acids and vitamins can also be tested.

Hypersensitivity can be demonstrated by skin testing with the fungal antigen, trichophyton. The reaction is known as **dermatophytid reaction.**

Treatment

This is by topical agents (ointments or gels) containing azoles (miconazole, clotrimazole, econazole) or terbinafine. Oral preparations of griseofulvin (dose: 100 mg, thrice daily), azoles (ketoconazole, itraconazole) or terbinafine may also be used.

Mild infections are treated by topical imidazole (clotrimazole or miconazole). **Severe infections** are treated by oral griseofulvin for 4–6 weeks. If hair is involved, treatment is given for 3–6 months. If nails are involved, treatment needs to be continued for one year. Alternative treatment consists of the administration of an oral imidazole (ketoconazole, 200 mg, twice daily), triazole (itraconazole, 100 mg, once daily; fluconazole, 150 mg, once daily) or terbinafine.

Epidemiology and prevention

Dermatophytoses occur throughout the world but certain types of disease and some species of fungi show geographically restricted distribution. Social and cultural patterns also influence dermatophytoses. Many factors such as age, hormones and intercurrent diseases affect the susceptibility of an individual to dermatophytoses.

Habitat

Depending on their natural habitat, dermatophytes may be classified as **anthropophilic**, **zoophilic** and **geophilic** species. Human beings are the main or only hosts for anthropophilic dermatophytes. *T. rubrum*, *E. floccosum* and *M. audouinii* are examples. They cause mild but chronic lesions. Zoophilic species are natural parasites of animals. Examples are *T. verrucosum* in cattle and
M. canis in dogs and cats. Human infections with zoophilic dermatophytes cause severe inflammation but are readily curable. Geophilic species, which occur naturally in soil, are relatively less pathogenic for human beings. Examples are *M. gypseum* and *T. ajelloi*.

Epidemics of tinea capitis may arise due to the use of shared barbershop hair clippers.

Control

This depends on personal cleanliness, avoiding contact with infectious material and effective treatment. Tinea pedis occurs only in people who wear socks with closed shoes. Candida infection of the nail is known as onychomycosis. This is often seen in persons who frequently come into contact with water and whose hands are often soggy. Details of the yeast-like fungus will be dealt with in detail in Chapter 57.

II. Subcutaneous Mycoses

These are principally seen in tropical and subtropical areas. The most frequent predisposing (risk) factor is trauma. The disease may remain localised or may spread by contiguity. Fungi that cause such subcutaneous mycoses are either normally present in the soil or are pathogens of plants. The outcome of such infections depends on fungal virulence and host defences. The various types include:

i) Mycotic mycetoma
ii) Chromomycosis (chromoblastomycosis and phaeohyphomycosis)
iii) Sporotrichosis
iv) Rhinosporidiosis
v) Subcutaneous phycomycosis (now referred to as entomophthoramycosis)

Mycotic Mycetoma

Clinical Case 2

A 45-year-old man, a farmer by occupation, presented to the Surgical OP with a history of multiple swellings in the foot and seropurulent pus discharge from the sinuses. The pus was subjected to Gram's stain, KOH preparation and modified acid-fast stain (to check for a botryomycotic, eumycotic or actinomycotic cause). KOH preparation revealed the presence of dark, pigmented, fungal hyphae, and Madurella mycetomatis was isolated on culture. The patient was treated with oral ketoconazole; debridement of the infected tissue with split graft was also performed (Fig. 56.16).

Fig. 56.16 Mycetoma of the foot with multiple swellings and seropurulent pus discharge from the sinuses (*Source:* Public Health Image Library, ID 21996 / Dr. Lucille K. Georg / CDC)

Mycetomas are chronic, slowly progressive, post-traumatic infections of the subcutaneous tissue caused by fungi and some bacteria found in the soil. They usually occur in the foot and lower extremities, and rarely infect other parts of the body. The disease was first reported by Gill (1842) from Madurai, South India. Carter (1860) established its fungal origin. It is commonly known as Maduramycosis or Madura foot. This has been referred to in the Atharva Veda as *padavalmika* (foot anthill).

Distribution

It is seen mainly in the tropics, though occasional cases have been reported from the temperate countries. In India, it is common in Tamil Nadu but rare in Kerala and northern parts of the country.

Causative agents and types

There are three types:

1. **Eumycetoma** (e.g., maduramycosis) caused by fungi namely, *Scedosporium* (*Pseudallescheria*), *Madurella mycetomatis* (Fig. 56.17), *M. grisea*, *Acremonium* spp., *Exophiala* spp., *Aspergillus nidulans* and *Fusarium* spp.
2. **Actinomycetoma** caused by actinomycetes namely, *Actinomadura*, *Streptomyces* and *Nocardia*.
3. **Botryomycosis** caused by *Staphylococcus aureus* and some other bacteria.

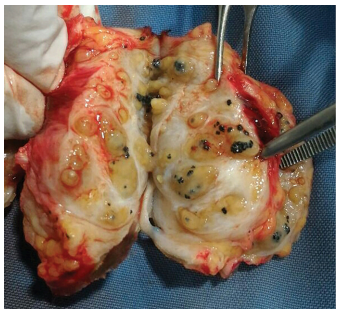


Fig. 56.17 Black granules in the tissue from a case of mycetoma (*Source:* Dr Angeline Radjou, Department of Surgery, Pondicherry Institute of Medical Sciences, Puducherry)

Epidemiology

The disease is endemic in regions with long dry seasons and short rainy spells, such as Central and South America, West and East Africa, India and Sri Lanka. Actinomycetoma occurs more commonly than eumycetoma. Persons engaged in agriculture are especially at risk.

Pathogenesis

The causative agent is believed to enter through minor trauma. The disease usually begins as a small subcutaneous swelling of the foot, which enlarges, burrowing into the deeper tissues and tracking to the surface as multiple sinuses discharging viscid, seropurulent fluid containing granules (Fig. 56.17). The lesions are painless (*Clinical case* 2).

Laboratory diagnosis

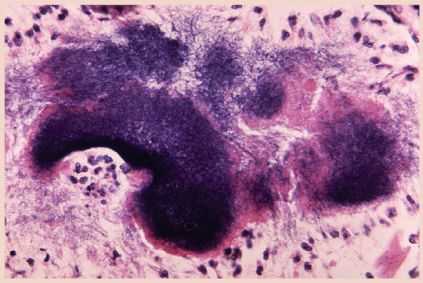
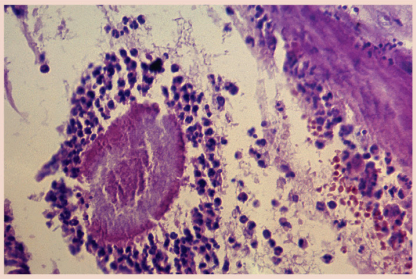
This is based on the examination of the granules from the sinus tracts on the swelling, culture of the granules and biopsy of the lesion.

Microscopy

The '**granules' or 'grains'** are microcolonies of the agents. The colour and consistency of the grains vary with the different agents causing the disease (Table 56.4). Compared to actinomycetoma, eumycotic lesions are harder, broader, may be pigmented, and often show septae and chlamydospores in KOH preparation. In actinomycotic mycetoma, on Gram stain, the actinomycetes appear as gram-positive, slender rods. On examination of the granules, they are seen to be composed of very thin (<1 μm in diameter) filaments, which are soft. Growth of organisms in culture on SDA at 37°C or room temperature, gross appearance of colony, rate of growth and typical microscopic appearance of hyphae (pigments), conidiogenous cells, conidia

and their arrangements help in establishing the diagnosis.

Table 56.4 Characteristics of eumycotic mycetoma and actinomycotic mycetoma

	Eumycetoma	Actinomycetoma
Causative agents	**Fungal etiology**	**Actinomycete (filamentous bacteria)**
Granules	Mostly **hard** in consistency (consist of tangled, tightly-packed fungal hyphae and tissue) **Brown to black** *Madurella mycetomatis, Madurella grisea, Exophiala jeanselmei* **White** *Scedosporium apiospermum* *Acremonium falciforme* Can be seen in KOH as pigmented or hyaline hyphae	**Soft in consistency** (consist of tissue elements and gram-positive filamentous bacilli) **White to yellow** *Nocardia asteroides* *Nocardia brasiliensis* *Actinomadura madurae* *Streptomyces somaliensis* **Red** *Actinomadura pelletierii*
Treatment	Surgical debridement, controlling secondary bacterial infection Antifungals: Topical nystatin or miconazole Itraconazole, fluconazole, amphotericin B (for Madurella infection)	Streptomycin, trimethoprim-sulphamethoxazole and dapsone
Histopathology Fig. 56.18 and 56.19	 **Fig. 56.18** Pigmented granule tissue stained by hematoxylin-eosin, reveals some of the histopathology associated with a case of black grain mycetoma, caused by the fungus *Madurella mycetomatis* (*Source:* Public Health Image Library, ID 14739 / Dr. Hardin / CDC).	 **Fig. 56.19** Hematoxylin-eosin (H and E) stained tissue specimen that had been extracted from a patient with an actinomycotic mycetoma revealed the presence of a sulphur granule. The actinomycosis was caused by the gram-positive bacterium, *Nocardia asteroides*. (*Source:* Public Health Image Library, ID 14745 / Dr. Libero Ajello / CDC)

Treatment

Surgery is the mainstay of treatment of eumycetoma and actinomycetoma.

Medical therapy for eumycetoma depends on the infecting fungus. *Scedosporium apiospermum* is treated with miconazole and ketoconazole; *M. mycetomatis*, with ketoconazole and griseofulvin; and *M. grisea* and *Fusarium*, by itraconazole. Resistant lesions may require amputation. Medical therapy for actinomycetoma consists of the use of dapsone (100 mg, thrice daily for 6–24 months), sulphonamides, cotrimoxazole or

rifampicin. **Therapy should be continued for 18–24 months even after the disappearance of clinical or laboratory evidence of infection.**

Chromomycosis

The term chromomycosis includes a group of clinical manifestations caused by various dematiaceous (pigmented) fungi.

Chromoblastomycosis (verrucous dermatitis)

This is the most common form of chromomycosis. The disease is mainly tropical and is more common among barefoot agricultural workers and woodcutters.

Clinical features

The lesions consist of warty, cutaneous nodules which resemble the florets of a cauliflower (Fig. 56.20). The disease is usually confined to the subcutaneous tissue of the feet and lower legs. The infecting fungi enter the skin by traumatic implantation. The lesion develops slowly around the site of implantation.

Fig. 56.20 Chromomycosis: Lesions that are warty, cutaneous nodules resembling the florets of a cauliflower (*Source:* Dr Sheela Kuruvila, Department of Dermatology, Pondicherry Institute of Medical Sciences, Puducherry)

Causative agents

The most common fungi responsible are species of the genera *Fonsecaea: F. pedrosoi,* and *F. compacta; Exophiala dermatitidis; Phialophora* (*P. verrucosa*) and *Cladophialophora* (*C. carrionii*). Infections caused by *F. pedrosoi* and *P. verrucosa* have been reported to disseminate to other areas, especially the brain.

Laboratory diagnosis

Histologically, the lesions show the presence of the fungus as round or irregular, dark brown, yeast-like bodies with septae, called **sclerotic bodies** (Fig. 56.21a and b). Diagnosis can be established by the demonstration of these sclerotic bodies in KOH mounts or in tissue sections, and by culture on Sabouraud agar.

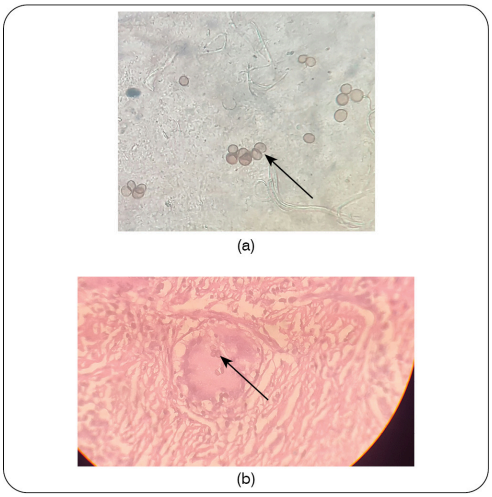


Fig. 56.21 Sclerotic bodies: (a) in KOH of tissue and (b) in the tissue section by H&E stain (*Source:* (a) Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry and (b) Department of Pathology, Pondicherry Institute of Medical Sciences, Puducherry)

Treatment

Amphotericin B, thiabendazole, 5-fluorocytosine, itraconazole and (recently) voriconazole have been found to be useful.

Phaeohyphomycosis

This group of **infections caused by dematiaceous fungi** includes localised or systemic infections caused by certain species such as *Phialophora, Cladosporium* or other dematiaceous soil fungi, showing brown filaments in the affected tissues. The sites of lesions may be cutaneous, subcutaneous, deeper tissues, or organs like the brain or lung. **Sclerotic bodies are not found.** The fungi appear in lesions as distorted hyphal strands. Phaeohyphomycosis is generally seen in debilitated or immunodeficient hosts. Some of the clinical types are:

- Brain abscess caused by *Cladophialophora bantiana* (formerly, *Cladosporium bantianum*)

- Subcutaneous or intramuscular lesions with abscesses or cysts containing masses of brown hyphae (formerly known as phaeosporotrichosis) caused by *Exophiala* (formerly, *Phialophora*) *jeanselmei, E. spinifera, E. dermatitidis* or *P. richardsiae.*

Sporotrichosis

This is a chronic infection involving cutaneous, subcutaneous and lymphatic tissue. It is frequently encountered in gardeners, forest workers and manual labourers. It may develop in otherwise healthy individuals. It occurs worldwide, but especially in Central and South America and South Africa.

Causative agent

Dimorphic fungus *Sporothrix schenckii* is the causative agent.

Pathogenesis and clinical presentation

- Lesions on the exposed parts of the skin follow minor trauma or thorn pricks. Nodules are first formed at the site of the lesion, followed by ulceration and necrosis of the nodules.
- From the lesions in the skin and subcutaneous tissue, the infection can spread via the lymphatic channels to the lymph nodes. This results in secondary ulcers on the lymph nodes, and the lymphatics are hardened and cord-like. This is 'lymphocutaneous' sporotrichosis.
- Systemic dissemination may occur to the bones, joints and meninges.

Laboratory diagnosis

1. **Specimens** The samples to be collected include aspirates from the nodules and biopsy material.

2. **Microscopy** Direct microscopic examination of KOH mounts of necrotic material or histopathological examination of tissue sections stained by methenamine silver stain shows a characteristic feature, the **asteroid body**: a rounded or oval, basophilic, yeast-like body, 3–5 μm in diameter, with

rays of an eosinophilic substance radiating from the yeast cell.

3. **Culture** Yeast cells are cultured on media incubated at 25ºC and 37ºC. *S. schenckii* is a dimorphic fungus occurring in the yeast phase in tissues and in cultures at 37ºC, and in the mycelia phase in nature and cultures at 25ºC. The septate hyphae are very thin (1–2 μm in diameter) and carry flower-like clusters of small conidia borne on delicate sterigmata (Fig. 56.22a and b).

Fig. 56.22 *Sporothrix schenkii*: (a) microscopic feature of thin hyphae with flower-like arrangement of microconidia and (b) lesions in the subcutaneous tissue, spreading via the lymphatics to the draining lymph nodes (*Source:* (a) Public Health Image Library, ID 21165 / Dr. Kaplan / CDC) and (b) Public Health Image Library, ID 16792 / Dr. Lucille K. Georg / CDC)

4. **Serology** Serological tests are especially helpful in the diagnosis of extracutaneous or systemic infection, where distinct clinical features are lacking. A slide latex agglutination test, using peptido-L-rhamno-D-mannan (outer layer of the fungal cell wall) as antigen, is a reliable, sensitive and specific test; results are obtained in minutes. A titre of 1:4 or greater is considered as presumptive evidence of sporotrichosis. In fixed cutaneous, subcutaneous, lymphocutaneous or systemic sporotrichosis, titres of 1:4 to 1:128 occur. A rising titre or sustained high titre is seen in pulmonary sporotrichosis. (Serological tests have poor prognostic value.)

Treatment

Spontaneous healing is possible.

- Potassium iodide, given topically or orally (250 mg thrice daily) for 2–4 months is effective in cutaneous infection.
- Itraconazole is effective in over 90 per cent of patients with lymphocutaneous infection; fluconazole (400 mg/day) and terbinafine (250 mg/day) can also be administered. Cryotherapy with liquid nitrogen is another treatment modality.
- For disseminated infection, amphotericin B is the drug of choice.

Rhinosporidiosis

This is a chronic granulomatous disease characterised by the development of friable polyps, usually confined to the nose, mouth or eye and, in rare cases, seen on the genitalia or other mucous membranes.

Distribution

Although the disease was first identified in Argentina, most cases have been reported from India and Sri Lanka.

Causative agent

The causative fungus is *Rhinosporidium seeberi*. The taxonomic position of this organism is currently uncertain, with doubts being raised about whether it can be considered a fungus.

Pathogenesis and clinical features

The mode of infection is not known, though infection is believed to originate from stagnant water or aquatic life.

Rhinosporidiosis is a localised condition which frequently presents as polypoidal growth in the nasal cavity; lesions can also be seen in the eye, skin, buccal cavity or genitalia.

While the disease is generally confined to mucous membranes, hematogenous dissemination has been recorded very rarely.

Laboratory diagnosis

Histologically, the lesion is composed of large numbers of fungal spherules embedded in a stroma of connective tissue and capillaries. The spherules are 10–200 μm
in diameter and contain thousands of endospores (Fig. 56.23a and b). *Rhinosporidium seeberi* has not been cultivated in artificial culture media.

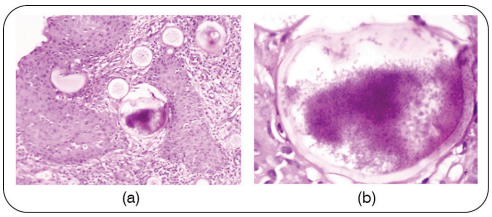


Fig. 56.23 Rhinosporidiosis: (a) tissue section showing sporangia with numerous endospores (10×) and (b) thick-walled sporangium with endospores (40×) (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Treatment

Excision of the polyp is the treatment of choice.

Subcutaneous Phycomycosis (Entomophthoromycoses)

Distribution

It was originally reported from Indonesia and, subsequently, identified in many Asian and African countries.

Causative agent

Conidiobolus coronatus and *Basidiobolus ranarum* are saprophytic zygomycetes found in decaying vegetation and in the intestines of many reptiles and amphibians.

Pathogenesis and clinical features

A painless subcutaneous nodule develops, which enlarges to involve a whole limb or large areas of the body. The lesions are now known to be acquired by insect bites
Fig. 56.24.

Fig. 56.24 Phycomycosis showing subcutaneous nodule which has enlarged to involve the whole upper limb (*Source:* Dr Sheela Kuruvila, Department of Dermatology, Pondicherry Institute of Medical Sciences, Puducherry)

Laboratory diagnosis

The causative fungi can be isolated by culture. Histopathological examination shows the presence of short, broad, fragmented hyphae with beak-like projections.

Treatment

The treatment of phycomycosis is difficult and surgery may be required. Antifungal drugs like itraconazole and terbinafine hydrochloride are used for two to three months following surgery.

Key points

- Fungi are eukaryotic Protista that possess a rigid cell wall containing chitin, mannan and other polysaccharides. They have true nuclei with nuclear membrane and paired chromosomes.
- Medically important fungi are classified based on their morphology as yeasts, yeast-like fungi, moulds (filamentous fungi) and thermally dimorphic fungi, while from the standpoint of disease-causing potential in humans, fungi are regarded as primary pathogens and opportunistic pathogens.
- Fungi are classified based on the method of sexual reproduction into Zygomycetes (asexual spores in sporangium), Ascomycetes (sexual spores within ascus), Basidiomycetes (sexual spores borne on basidium) and Deuteromycetes ('fungi imperfecti' with no known sexual form).
- Pathogenic fungi cause true infections or mycoses, mycotoxicoses and allergic diseases (Type I and Type III hypersensitivity reactions).
- Superficial mycoses include pityriasis versicolor, tinea nigra and black and white piedra.
- Cutaneous infections include dermatophytosis (caused by three genera of dermatophytes: *Trichophyton, Microsporum* and *Epidermophyton*). Dermatophytosis manifests as ringworm (tinea). Dermatophytes grow relatively slowly on laboratory culture media at room temperature.
- Subcutaneous mycoses are fungal infections of subcutaneous tissues. They remain localised or spread by contiguity. The main types are mycotic mycetoma (eumycetoma), chromoblastomycosis, phaeohyphomycosis, sporotrichosis, rhinosporidiosis and entomophthoromycosis.
- Mycetoma (maduramycosis, Madura foot) occurs worldwide. The causative agents are bacteria (cause actinomycetoma and botryomycosis) and fungi (cause eumycetoma) found in soil or plants. The colour and texture of granules ('grains') from lesions is of diagnostic value.

- Sporotrichosis is a subcutaneous granulomatous infection caused by *Sporothrix schenckii* (thermally dimorphic fungus). It remains confined to the skin (fixed cutaneous form) or involves the local lymphatics. It commonly follows trivial trauma from thorns or wood splinters. The asteroid body is of diagnostic relevance.
- Chromoblastomycosis (chromomycosis) is a chronic localised mycosis of skin and subcutaneous tissues of the limbs. It is characterised by raised, crusted lesions and is caused by several brown-pigmented (dematiaceous) fungi. Sclerotic bodies in the infected tissues are of diagnostic relevance.
- Rhinosporidiosis is a chronic granulomatous disease characterised by friable polyps, usually confined to the nose, mouth or eye. It is caused by *Rhinosporidium seeberi* (hitherto not cultivated in culture).
- Entomophthoramycosis is characterised by painless subcutaneous nodules which enlarge to involve the entire limb or large areas of the body. It is caused by *Conidiobolus coronatus* and *Basidiobolus ranarum.* The lesions are acquired by insect bites.

Questions

Essays

1. Describe the pathogenesis and laboratory diagnosis of dermatophytes.
2. Enumerate the fungi that cause subcutaneous mycoses. Describe the pathogenesis and laboratory diagnosis of eumycotic mycetoma.

Short Answers

1. Piedra
2. Superficial mycosis
3. Pityriasis versicolor
4. Tinea corporis
5. Favus
6. Infections caused by *Trichophyton* species
7. Laboratory diagnosis of dermatphytes
8. Rhinosporidiosis

CHAPTER 57

Systemic Fungal Infections

Chapter navigation

- Deep mycoses
- Opportunistic mycoses
- Mycotoxins

INTRODUCTION

Systemic mycoses refer to disseminated or deep fungal infections not restricted to the superficial areas. They are caused by fungi that are mostly soil saprophytes and are endemic to certain geographic areas. Systemic mycoses occur in varying degrees of severity, ranging from asymptomatic infection to fatal disease.

The fungi causing systemic mycoses are **dimorphic** (they are in the yeast form at 37°C and mould at 25°C). Some of the fungi causing systemic infections are:

1. *Histoplasma capsulatum*
2. *Blastomyces dermatitidis*
3. *Paracoccidioides brasiliensis*
4. *Coccidioides immitis*

A. SYSTEMIC MYCOSES (DIMORPHIC FUNGI)

HISTOPLASMOSIS

Histoplasmosis is an infection of the reticuloendothelial system caused by the thermally dimorphic fungus *Histoplasma capsulatum*. The disease was originally described by Darling (1905) and came to be known as Darling's disease. He believed the causative agent to be a protozoon related to *Leishmania donovani*.

Distribution

The disease occurs worldwide; however, it is most common in the USA where it is endemic in many central and eastern states. In endemic areas, the fungus is present in the soil, decaying trees and is particularly abundant in bird droppings. *Histoplasma capsulatum* is differentiated into two varieties:

- *Histoplasma capsulatum* var. *capsulatum*, which causes the classical, ubiquitous form of histoplasmosis
- *Histoplasma capsulatum* var. *duboisii*, which causes 'African histoplasmosis'

Clinical Case 1

A 35-year-old farm worker, who worked with chicken coops, presented with fever, cough, anorexia and lymphadenopathy. X-ray of the chest showed focal infiltrates and patchy opacities. Aspiration from his lymph nodes showed the presence of intracellular yeast. Culture incubated at 25°C and at 37°C yielded yeast forms at 37°C and mould at 25°C. The mycelial form showed the presence of thick-walled, spherical spores with tubercles (tuberculate macroconidia) and microconidia. The fungus was identified as H. capsulatum. The patient was put on liposomal amphotericin B for a period of two weeks following which he responded well to therapy.

Pathogenesis and Clinical Features

Infection is acquired by inhalation. Most infections of classical histoplasmosis are asymptomatic and heal spontaneously, leaving behind an area of miliary calcification (*Clinical case* 1). Some infected individuals develop pulmonary disease which resembles tuberculosis. Disseminated histoplasmosis develops only in a minority of infected individuals. Infection of the reticuloendothelial system manifests as lymphadenopathy, hepatosplenomegaly, fever and anemia with a high rate of fatality (Fig. 57.1). It is an intracellular granulomatous lesion that is ulcerative. It may also develop on the skin and mucous membranes.

African histoplasmosis involves mainly the skin, subcutaneous tissues and bones. The lungs are not commonly affected and disseminated disease is infrequent.

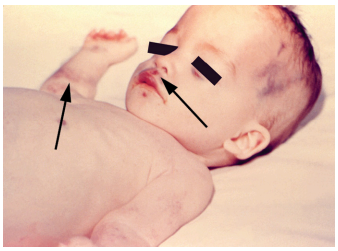


Fig. 57.1 Disseminated histoplasmosis in a child (*Source:* Public Health Image Library, ID 15361 / Dr. Lucille K. Georg / CDC)

Laboratory Diagnosis

1. **Microscopy** Diagnosis of histoplasmosis involves the microscopic examination of stained smears of blood, bone marrow, scrapings from lesions or biopsies of lymph nodes. In classical histoplasmosis, small intra- and extracellular yeast cells are seen in Giemsa- or Wright-stained smears of infected tissues. In tissues, the yeast phase occurs within phagocytic cells. The yeast appears as an oval, budding cell measuring 2–4 μm (Fig. 57.2a and b), while in African histoplasmosis, much larger yeast-like cells (7–15 μm) are seen.

2. **Culture** The yeast phase also grows out in culture on blood agar at 37°C. On Sabouraud or other agar plates at room temperature, white, cottony, mycelial growth appears, with large (8–20 μm) thick-walled, spherical spores with tubercles (finger-like projections) (Fig. 57.2c). These **'tuberculate' spores** are of diagnostic relevance; the mycelial phases of both varieties are indistinguishable.

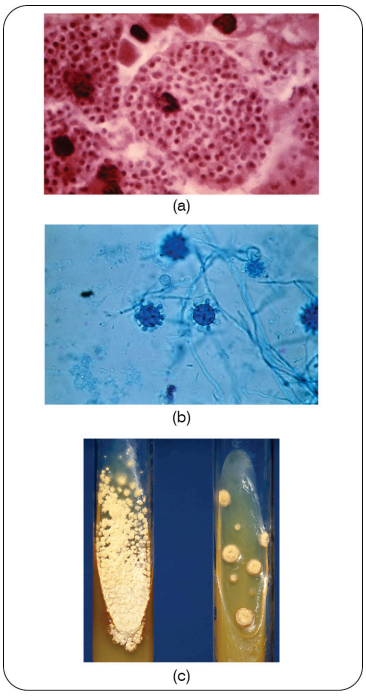


Fig. 57.2 *Histoplasma capsulatum*: (a) yeast form (intracellular); (b) mycelial form at 37°C and (c) slant cultures of *H. capsulatum* colonies on Sabouraud agar (*Source:* (a) Public Health Image Library, ID 14422 / Armed Forces Institute of Pathology / CDC; (b) Public Health Image Library, ID 15364 / Dr. Libero Ajello / CDC and (c) Public Health Image Library, ID 4189 / Dr. Lenore Haley / CDC)

3. **Serodiagnosis** Antibodies formed during the course of systemic mycoses increase in titre in progressive disease. Hence serological tests such as latex agglutination, complement fixation and precipitation tests are useful in the diagnosis of histoplasmosis. **Titre is considered positive at reciprocal dilutions greater than 1:8. A titre with dilutions greater than 1:32 suggests active histoplasmosis infection.**

Antigens can be detected in urine and serum. These tests are useful in immunocompromised individuals in whom antibody formation is impaired.

4. **Skin tests** Delayed hypersensitivity develops following infection. It can be demonstrated by skin testing with **'histoplasmin'**, which is analogous to the tuberculin test for tuberculosis. In histoplasmosis, skin tests are more specific than serological methods.

BLASTOMYCOSIS

This is a chronic infection, characterised by the formation of suppurative and granulomatous lesions with a marked predilection for the lungs and

skin. It can also occur in any other part of the body.

Distribution

The infection is largely confined to North America; hence it is known as North American blastomycosis (to be differentiated from paracoccidioidomycosis, which is also known as South American blastomycosis). In recent years, several cases have been reported from Africa and India. The fungus has also been isolated in Delhi from the bronchial aspirates of a patient and from the lungs of insectivorous bats.

Causative Agent

Blastomycosis is caused by the thermally dimorphic fungus *Blastomyces dermatitidis* [teleomorph (perfect state) is *Ajellomyces dermatitidis*].

Pathogenesis and Clinical Features

Infection is acquired by the inhalation of contaminated soil which is the habitat.

- **Primary infection** of the lungs may resemble tuberculosis or histoplasmosis. The condition may be asymptomatic or may lead to focal or diffuse consolidation, miliary lesions or abscess formation.
- **Disseminated disease** may develop through the bloodstream to form multiple abscesses in various parts of the body. The fatality rate is high in this form of the disease.
- **Cutaneous disease** usually affects the skin of the face or other exposed parts of the body. The initial lesion is a papule, around which secondary nodules develop and coalesce (Fig. 57.3).

Fig. 57.3 Large, elevated ulcerative lesions in blastomycosis (*Source:* Public Health Image Library, ID 14873 / Dr. Lucille K. Georg / CDC)

Laboratory Diagnosis

Culture In tissues and in culture at 37°C, the fungus appears as a budding yeast cell, which is large (7–20 μm) and spherical, with a thick, double-contoured wall. Each cell carries only a single broad-based bud. At room temperature, the culture is filamentous with septate hyphae and many round or oval conidia, and, in older cultures, chlamydospores are also seen (Fig. 57.4a and b). In tissues, single budding yeast cells with broad bases are seen (Fig. 57.4c).

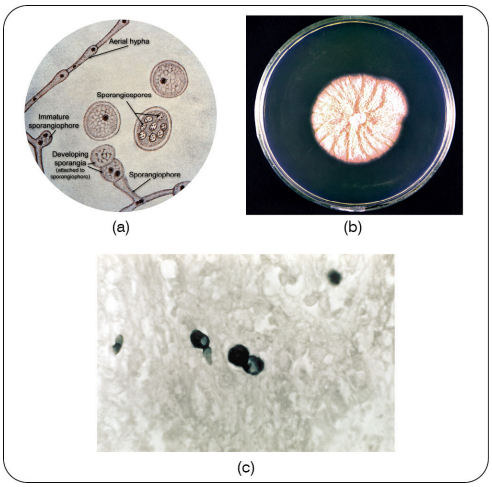


Fig. 57.4 (a) Mycelial and yeast forms; (b) culture of *Blastomyces dermatitidis* and (c) **histopathology of tissue section in blastomycosis by methenamine silver stain; shows broad-based, single budding cell** (*Source:* (a) Public Health Image Library, ID 14355 / CDC; (b) Public Health Image Library, ID 19914 / Dr. Libero Ajello / CDC and (c) Public Health Image Library, ID 4226 / Dr. William Kaplan / CDC)

PARACOCCIDIOIDOMYCOSIS

This is a chronic granulomatous disease of the skin, mucosa, lymph nodes

and internal organs.

Distribution

As the disease is confined to South America, it is called South American blastomycosis. It occurs in rural areas.

Causative Agents

Paracoccidioidomycosis is caused by the thermally dimorphic fungus, *Paracoccidioides brasiliensis*.

Pathogenesis and Clinical Features

The spores are inhaled and result in primary pulmonary infection that spreads by the hematogenous route to the mucosa of the nose, mouth, gastrointestinal tract, skin and lymphatics. This leads to ulcerative granulomas of the buccal and nasal mucosa, which are a prominent feature of this disease (Fig. 57.5a)

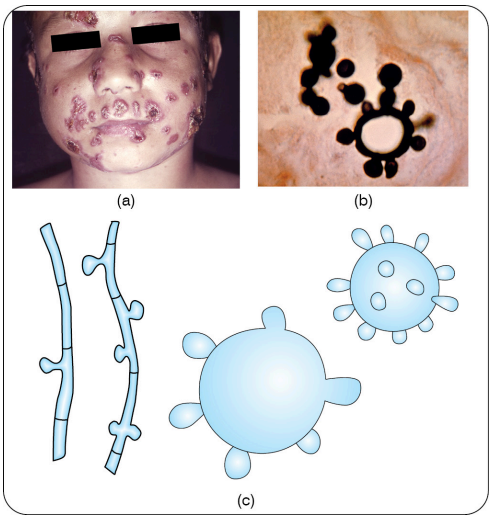


Fig. 57.5 (a) Lesions on the face due to paracoccidioidomycosis; (b) budding yeast form in methenamine silver stain of tissue and (c) mycelial and yeast forms (*Source:* (a) Public Health Image Library, ID 12156 / Dr. Martins Castro, San Paulo, Brazil; Dr. Lucille K. Georg / CDC and (b) Public Health Image Library, ID 527 / Dr. Lucille K. Georg / CDC)

Laboratory Diagnosis

1. **Microscopy** The yeast phase occurs in tissues and in cultures at 37°C as large, globose or oval cells with multiple buds encircling the mother cell;

this **mariner's wheel** or **pilot's wheel** appearance is characteristic (Fig. 57.5 b).

2. **Culture** The mycelial phase is found in nature and can be isolated in culture after incubation at 25–30°C for two to three weeks (Fig. 57.5c).

COCCIDIOIDOMYCOSIS

Coccidioidomycosis is a primary pulmonary infection that may be inapparent, benign, severe or even fatal.

Distribution

The disease is endemic in the dry, arid regions of southwestern USA, where the fungus is present in the soil and in rodents.

Causative Agents

Coccidioidomycosis is caused by the thermally dimorphic fungus *Coccidioides immitis* (Fig. 57.6).

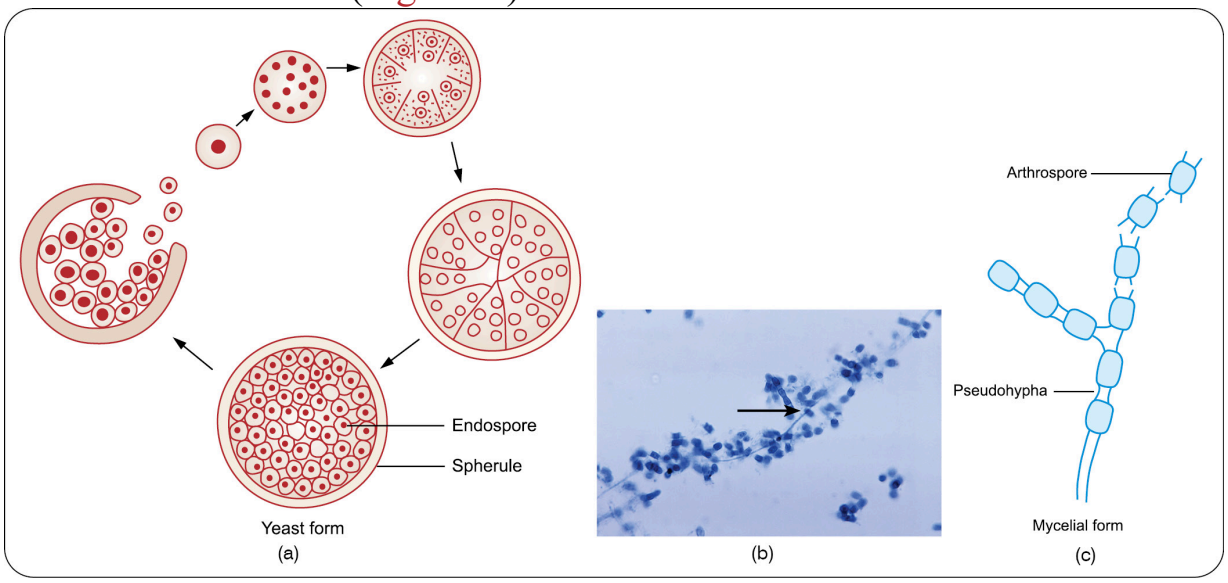


Fig. 57.6 *Coccidioides immitis:* (a) spherules; (b) arthroconidia and (c) thick-walled arthroconidia (*Source:* (b) Public Health Image Library, ID 12201 / Lucille Georg / CDC)

Pathogenesis and Clinical Features

Infection is acquired by the inhalation of dust containing arthrospores of the fungus. In most cases, the respiratory infection is asymptomatic and leads to lifetime immunity.

A self-limited influenza-like fever (known as **valley fever or desert rheumatism**) may occur in some individuals. Less than one per cent of infected persons develop the chronic progressive disseminated disease, coccidioidal granuloma (Fig. 57.7a), which is highly fatal.

Laboratory Diagnosis

1. **Culture** The fungus is thermally dimorphic, occurring as a spherule in tissues and in culture at 37°C and as the mycelial form in soil and in culture at room temperature. The spherule is 15–75 ∝m in diameter, with a thick, doubly refractile wall and filled with endospores (Fig. 57.7a). The mycelial phase consists of hyphae which fragment into arthrospores that are highly infectious (Fig. 57.7b and c). Culture confirmation can be performed rapidly and with less risk to staff by DNA probe or exoantigen testing in a biological safety cabinet.

2. **Skin test** Delayed hypersensitivity can be demonstrated by a positive skin test with **coccidioidin** (analogous to the tuberculin test).

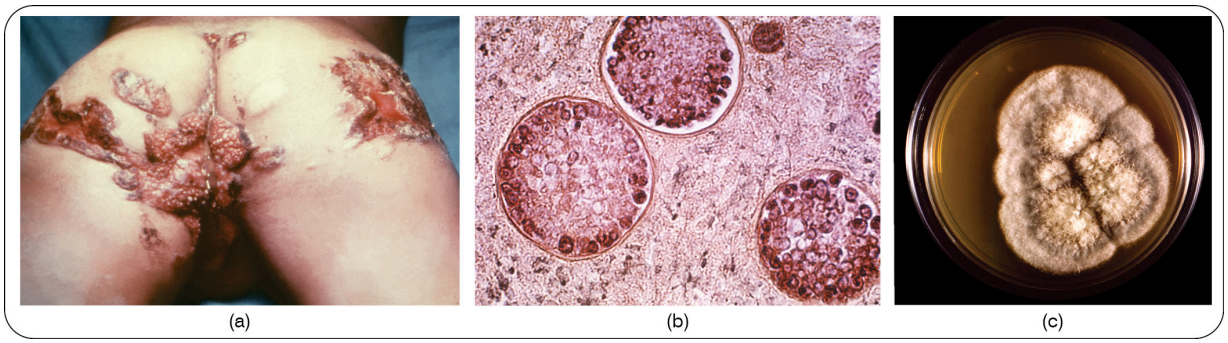


Fig. 57.7 (a) Coccidioidomycosis lesions on the patient's buttocks; (b) large spherule in tissue with endospores and (c) grayish-white downy culture of *Coccidiodes immitis* (*Source:* (a) Public Health Image Library, ID 20552 / Dr. Brodsky / CDC; (b) Public Health Image Library, ID 14641 / Dr. Lucille K. Georg / CDC and (c) Public Health Image Library, ID 21608 / Dr. Libero Ajello / CDC)

TREATMENT OF SYSTEMIC MYCOSES

Liposomal amphotericin B is the drug of choice for severe (invasive) disease. Oral azoles (ketoconazole, itraconazole, fluconazole and, more recently, voriconazole) are used for less severe infections. For paracoccidioidomycosis, oral itraconazole 50–100 mg/day is the drug of choice; ketoconazole and fluconazole are less effective. Fluconazole is

useful for coccidioidal meningitis. Corrective surgery may be used for pulmonary and cutaneous lesions.

B. OPPORTUNISTIC MYCOSES

Opportunistic mycoses develop in patients who are immunosuppressed, those who have hematological malignancies or diabetes, or who are on immunosuppressive drugs, corticosteroids, radiotherapy or broad-spectrum antibiotics. Opportunistic mycoses are caused by fungi that are of low virulence and found as contaminants in the environment (e.g., *Mucor*, *Penicillium*, *Aspergillus)* or as commensal flora of the body (e.g., *Candida*).

ASPERGILLOSIS

Aspergillus is the commonest mould seen on damp surfaces or almost any other organic matter. Their habitat is also soil and dust. Their spores are ubiquitous. Of the 300-odd species of *Aspergillus, A. fumigatus* is the main pathogen. It causes invasive disease in immunocompromised human beings. Other species associated with infection include *A. niger*, *A. flavus* and *A. nidulans*.

Pathogenesis and Clinical Features

Aspergillus species can cause a variety of clinical syndromes:

1. ***Allergic bronchopulmonary aspergillosis*** Inhaled spores provoke a hypersensitivity reaction, which may be:

    - Type I hypersensitivity (asthma), which occurs in atopic individuals following sensitisation to inhaled aspergillus spores
    - Type III hypersensitivity (extrinsic alveolitis)
    - Combined Type I and Type III hypersensitivity reactions

    In bronchopulmonary aspergillosis, the fungus grows within the lumen of the bronchioles, which may be occluded by fungal plugs; the fungus can be demonstrated in sputum. The condition is made worse by the development of hypersensitivity to the fungus.
2. ***Aspergilloma*** Here, a fungal ball grows within and is usually restricted to an existing lung cavity, for example, due to old

tuberculosis or bronchiectasis. In this type of **colonising aspergillosis**, surgical removal becomes necessary because the disease commonly causes massive hemoptysis.

3. ***Invasive aspergillosis*** Here, the fungus first causes pneumonia and later disseminates to involve other organs, for example, the brain, kidneys or heart. Patients who develop this type of disease, which may be fatal, are usually immunocompromised or debilitated due to prolonged treatment with antibiotics, steroids and cytotoxic drugs.
4. ***Superficial infections*** *Aspergillus* species can cause infections of the external ear (otitis externa)—otomycosis; niger and favus are the species involved. Secondary infections also affect the eye (mycotic keratitis) and nasal sinusitis.

Laboratory Diagnosis

Demonstration of septate hyaline hyphae from tissues or specimen by direct microscopy is suggestive of *Aspergillus* infection but this is not diagnostic.

1. **Specimen**

i. **Exudates, bronchial washings and bronchoalveolar lavage**—samples are first examined for the presence of fungal elements by wet mount preparation in 10% potassium hydroxide. Repeat specimens to demonstrate similar findings must be done for confirmation.

ii. **Tissue sections**—for example, tissue sections of biopsy or post-mortem material are stained by the periodic acid Schiff method. The hyphae are poorly stained by hematoxylin and eosin. Typical dichotomous branching at acute angles is characteristic of aspergillus infection (Fig. 57.8).

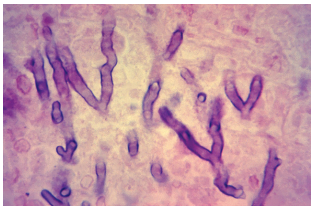


Fig. 57.8 H and E photomicrograph of a human lung tissue showing dichotomous branching hyphae (*Source:* Public Health Image Library, ID 15630 / Armed Forces Institute of Pathology (AFIP); Dr. Hardin / CDC)

iii. **Detection of galactomannan** (a component of the *Aspergillus* cell wall) in serum or bronchoalveolar lavage fluid is a marker for the

diagnosis of invasive aspergillosis in adults and children, in hematopoietic stem cell transplant recipients or in patients with hematologic malignancies.

iv. Colonies in culture grow after 48 hours but longer incubation may be required before characteristic morphological features develop.

v. After 3–4 days' incubation on **Sabouraud agar** at 25–37°C, the colonies have a velvety to powdery surface and are characteristically coloured: *A. fumigatus*—dark green, *A. niger*—black and *A. flavus*—yellow-green (Fig. 57.9a, b and c).

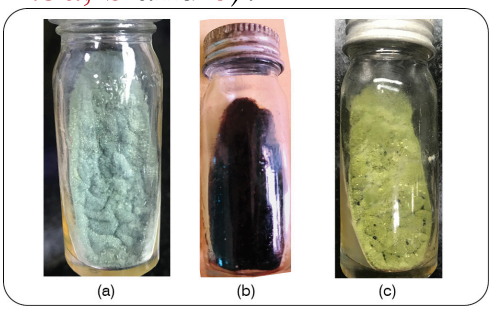


Fig. 57.9 *Aspergillus* colonies on Sabouraud agar
(a) *A. fumigatus*; (b) *A. niger* and (c) *A. flavus* (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

2. **Microscopic appearance of the colony** A wet preparation stained with lactophenol cotton blue demonstrates septate hyphae and conidiophores (specialised aerial hyphae that bear spores or conidia). The conidiophores have swollen, rounded ends ('vesicles') with chains of conidia borne on elongated cells called sterigmata. The general morphology is characteristic of the genus and there are also inter-species differences that are useful in identification (Fig. 57.10a, b and c).

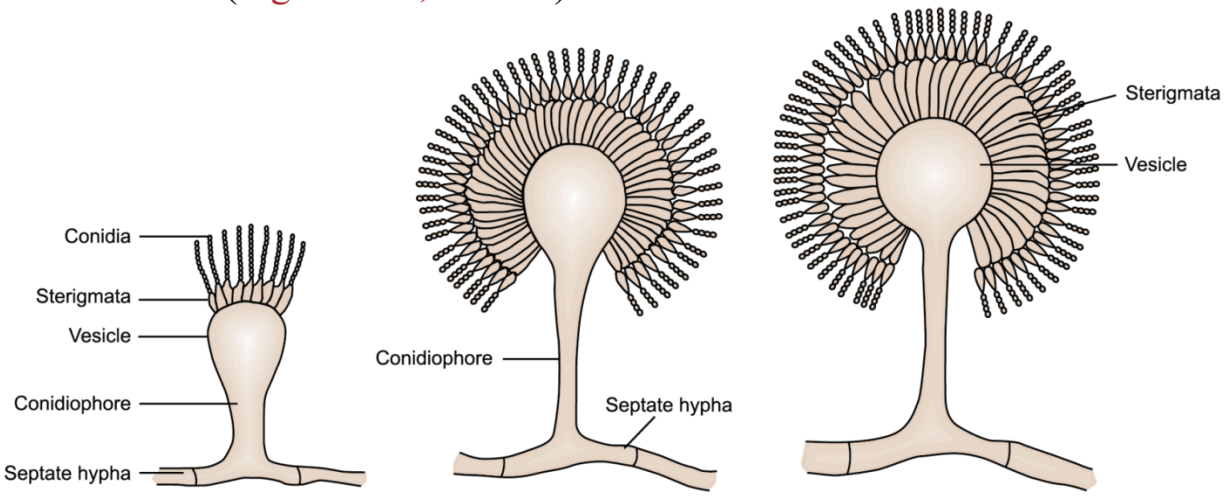


Fig. 57.10 *Aspergillus* spp.; (a) *A. fumigatus*; (b) *A. flavus* and (c) *A. niger*

3. **Serology** Precipitating antibodies to aspergillus antigens can be demonstrated by countercurrent immunoelectrophoresis, immunodiffusion and enzyme-linked immunosorbent assay (ELISA). Antibodies are usually absent in the sera of healthy individuals: they can be detected in the majority (70 per cent) of patients with allergic aspergillosis and approximately the same proportion of those with pneumonia or invasive disease.

4. **Polymerase chain reaction and nucleic acid sequence-based amplification** These are other advanced methods for establishing the diagnosis of aspergillosis tracheobronchitis.

5. **Skin tests** **Dual immediate** (after 15 minutes) and **Arthus type** (after 4–6 hours) skin test reactions which develop after the intradermal injection of *Aspergillus* spp. antigens are important criteria to establish a diagnosis of allergic broncho-pulmonary aspergillosis.

Treatment

Invasive aspergillosis is treated with intravenous amphotericin B/liposomal amphotericin B. However, the mortality is high. Intravenous formulations of azoles (voriconazole, itraconazole and posaconazole) are currently used.

PENICILLOSIS

Penicillium is present in the environment and grows on various substrates such as bread, jam, fruit and cheese. These organisms cause opportunistic infections in diabetics. In the laboratory, they are common airborne contaminants of culture media. Colonies are blue-green in colour with a white border and a powdery surface (Fig. 57.11b). Microscopy demonstrates septate hyphae with branched conidiophores, with two rows of sterigmata bearing chains of spores; the appearance is like a brush or broom (Fig. 57.11a). *Penicillium* may cause opportunistic mycoses.

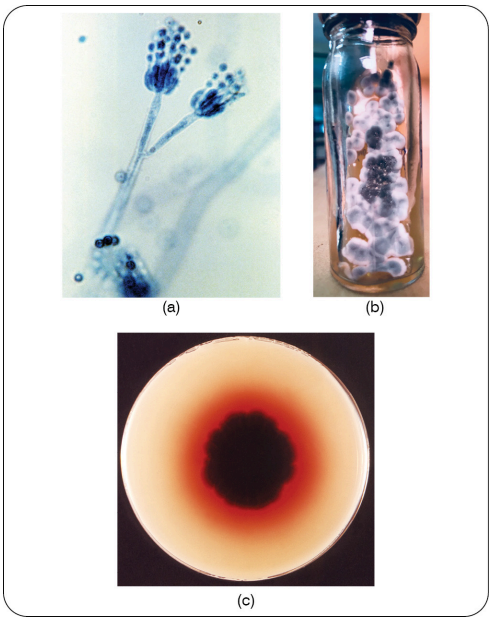


Fig. 57.11 (a) *Penicillium*: Chains of conidia produced by phialides, which are supported by branched conidiophores, terminal conidium being the oldest; (b) blue-green colonies of *Penicillium* on Sabouraud dextrose agar and (c) red diffusible pigment on the reverse of *Penicillium marneffei* colony (*Source:* (a) Public Health Image Library, ID 8398 / Lucille Georg / CDC, (b) Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry and (c) Public Health Image Library, ID 11967 / Dr. Libero Ajello / CDC)

Pathogenesis and Clinical Features

P. marneffei, now known as ***Talaromyces marneffei*,** is endemic in South-East Asia, southern China and North-Eastern India. It affects immunocompromised patients—those with HIV hematological malignancy and those undergoing immunosuppressive therapy. Bamboo rats are a natural reservoir. Infection is associated with high humidity and exposure to plants and farm animals. The clinical symptoms range from mild to moderate infection with localised disease, to disseminated infection with multiple organ involvement, or severe disease including respiratory failure and shock. They may invade the gastrointestinal tract with oropharyngeal ulcerations and diarrhea. Occasionally, the central nervous system is involved, leading to meningoencephalitis. This fungus is unique among *Penicillium* in that it is a true pathogen.

Laboratory Diagnosis

Microscopy of skin scraping, sputum smears, or biopsies shows binary

fission yeasts on fungal staining. The yeast forms are small, oval and 2–4 µm in diameter. The mycelial form produce a red diffusible pigment (Fig. 57.11c) and morphologically resembles other members of the *Penicillium* species.

Treatment

The infection can be treated with amphotericin B, followed by oral itraconazole.

ZYGOMYCOSIS (MUCORMYCOSIS, PHYCOMYCOSIS)

Zygomycosis (commonly referred to as **mucormycosis**) is an invasive disease caused by zygomycetes (phycomycetes), principally of the species of *Rhizopus*, *Mucor* and *Absidia.* These fungi are ubiquitous in soil and their spores are present in air and dust. They often contaminate stale bread.

Pathogenesis of Infection and Clinical Features

Zygomycosis develops as a systemic infection following dissemination from a primary focus in the upper respiratory tract or nasal cavity, where the spores germinate and the mycelia invade the adjacent tissues. Angio-invasiveness is one of the characteristics of zygomycosis which makes it fatal in invasive infections—the orbit, sinuses and the brain; the lung may also be the primary site of infection. Almost all patients are immunocompromised. The rhinocerebral (or rhino-orbito-cerebral) (Fig. 57.12a) form, in which the nose, nasal sinuses and orbit are involved, is well-recognised and is usually a fatal complication of diabetes mellitus. When the lung is the primary site of infection, the fungi may invade the arteries to cause thrombosis and infarction.

The incidence of the disease has increased considerably as a result of the widespread use of antibacterial antibiotics, corticosteroids and antimetabolites.

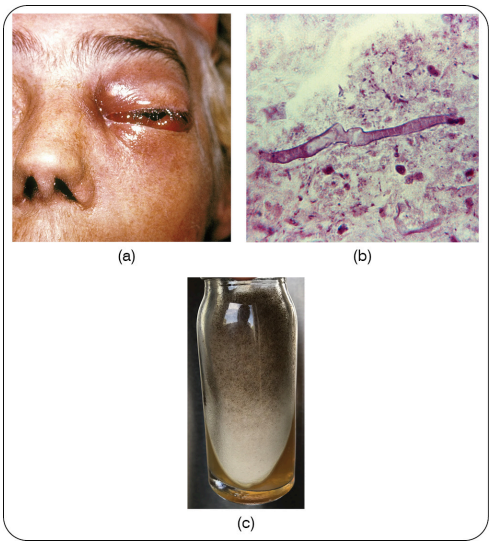


Fig. 57.12 (a) Zygomycotic infection of the upper eye lid; (b) broad ribbon-like aseptate hypha of the fungus and (c) cottony appearance of the colony of *Mucor (Source:* (a) Public Health Image Library, ID 2831 / Dr. Thomas F. Sellers / Emory University / CDC; (b) Public Health Image Library, ID 14550 / Dr. Lucille K. Georg / CDC and (c) Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

Laboratory Diagnosis

Diagnosis is usually made during histological examination of autopsy material by noting the presence of broad and bizarre, non-septate (or sparsely septate) hyphae in tissues.

1. **Specimen**

Exudate A wet preparation is made in 10% potassium hydroxide. Broad, ribbon-like hyphae are seen.

Tissue Hyphae stain readily with hematoxylin and eosin (unlike aspergillus) showing non-septate, broad hyphae, often invading the blood vessels (Fig. 57.12b).

2. **Culture** Isolation is by culture on Sabouraud agar with an antibacterial agent but without cycloheximide, on which the fungi grow easily (however, growth may sometimes be difficult to achieve from necrotic material even when abundant hyphae are seen). Culture characteristics of the main genera are similar.

After incubation for 3–4 days on Sabouraud agar at 30–37°C, the colonies are grey-white with a thick, cottony, fluffy surface (Fig. 57.12c). Under the microscope, non-septate, broad hyphae with aerial sporangiophores which

end in a sporangium (a sac containing spores) are seen.

Mucor shows branched sporangiophores arising randomly along aerial mycelium. Rhizoids are absent. Rhizopus has rhizoids, and sporangiophores arise in groups directly above the rhizoids (Fig. 57.13a and b).

Note: These common environmental moulds are frequent contaminants of culture plates.

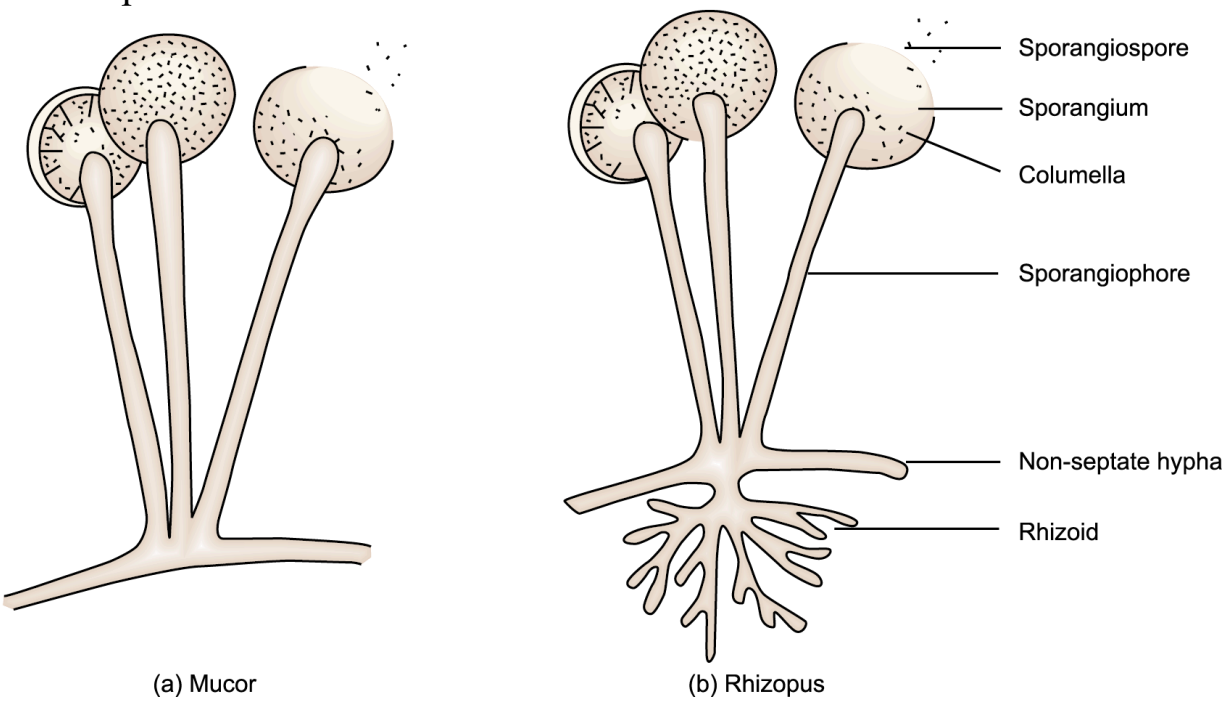


Fig. 57.13 Zygomycetes: (a) *Mucor* and (b) *Rhizopus*

Treatment

Intravenous amphotericin B combined, where appropriate, with surgical drainage is the recommended treatment. Good medical control of diabetes is required.

CANDIDOSIS (CANDIDIASIS/MONILIASIS)

Candidosis refers to an infection of the skin, mucosa, and rarely, of the internal organs, caused by a yeast-like fungus, *Candida albicans*. Other *Candida* species are being increasingly reported from clinical infections. Candidosis is an opportunistic endogenous infection, the commonest predisposing factor being diabetes.

Distribution

Candida species are normal inhabitants of the skin and mucosa.

Causative Agent

Candida albicans is an ovoid or spherical budding cell, which produces pseudomycelia both in tissues and in culture (Fig. 57.14a and b).

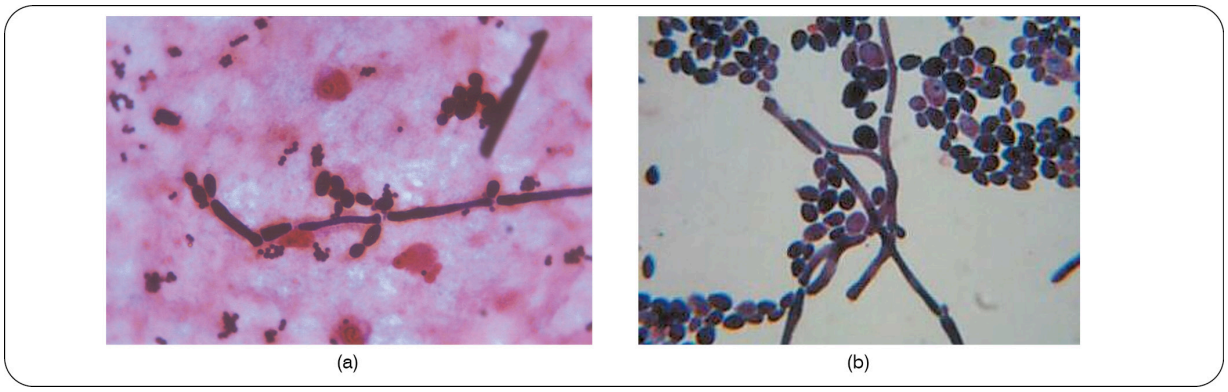


Fig. 57.14 Gram stain showing gram-positive: (a) *Candida* yeast with pseudohyphae and pus cells in tissue and (b) *Candida* yeast with pseudohyphae in culture

***Candida* species of growing clinical importance**
Other *Candida* species involved in human infection include *C. glabrata, C. tropicalis, C. keyfr (*formerly, *C. pseudotropicalis*), *C. krusei, C. guilliermondii, C. parapsilosis* and *C. stellatoidea. Candida auris* is a recent finding globally, seen to be resistant to many antifungal agents. It is associated with fatal infections acquired in the hospitals. It has also been reported from India.

Pathogenesis and Clinical Features

Cutaneous candidosis This may be intertriginous or paronychial. The former is an erythematous, scaling or moist lesion with sharply demarcated borders, where papular lesions are most prominent. The sites affected are those where the skin is macerated by perspiration: the groin, perineum, axillae and inframammary folds. **Paronychia** and **onychomycosis** are seen in occupations that involve frequent immersion of the hands in water.

Mucosal lesions Common lesions are **vaginitis**, characterised by an acidic discharge and found frequently in pregnancy, and **oral thrush**, found commonly in bottle-fed infants and aged, immunocompromised and debilitated persons (Fig. 57.15). In these conditions, creamy white patches appear on the tongue or buccal mucosa; following removal, these leave a red, oozing surface. This is also seen in HIV and other

immunocompromised individuals. Oesophageal candidosis is now considered an AIDS-defining illness.

Fig. 57.15 Oral thrush due to *Candida albicans* in an immunocompromised patient (*Source:* Public Health Image Library, ID 6067 / John Molinari, Ph.D., University of Detroit, Detroit, Michigan; Sol Silverman, Jr., D.D.S. / CDC)

Intestinal candidosis This is a frequent sequel to excessive oral antibiotic therapy and may present as diarrhea not responding to antibacterial treatment.

Bronchopulmonary candidosis This is seen as a rare complication of pre-existing pulmonary or systemic disease.

Candida has now emerged as a hospital-acquired pathogen in catheterised patients. **Catheter-associated UTI or bloodstream infections** are caused due to catheter colonisation.

Systemic infections such as septicemia, endocarditis and meningitis may occur as terminal complications in severe generalised diseases such as leukemia and in persons on prolonged immunosuppression. Candida granuloma and chronic mucocutaneous candidosis are serious manifestations seen in immunodeficiencies.

Laboratory Diagnosis

This can be established by microscopy and culture.

1. **Microscopy** Wet films or Gram-stained smears from lesions or exudates show budding gram-positive cells (Fig. 57.16a). *Candida* can colonise normal skin or mucosa as well. Abundant presence is of significance. Demonstration of mycelial forms indicates colonisation and tissue invasion and is, therefore, of greater significance.

2. **Cultures** *Candida* grows readily on **Sabouraud agar** and on ordinary bacteriological culture media. Colonies are creamy white, smooth and have a yeasty odour (Fig. 57.16b). *Candida albicans* can be differentiated from other *Candida* species by growth characteristics, sugar assimilation and

fermentation tests. *C. albicans* alone forms **chlamydospores** on cornmeal agar cultures at 20°C. A rapid method of identifying *C. albicans* is based on its ability to form **germ tubes** within two hours when incubated in human serum at 37°C (Reynold–Braude phenomenon) (Fig. 57.16c).

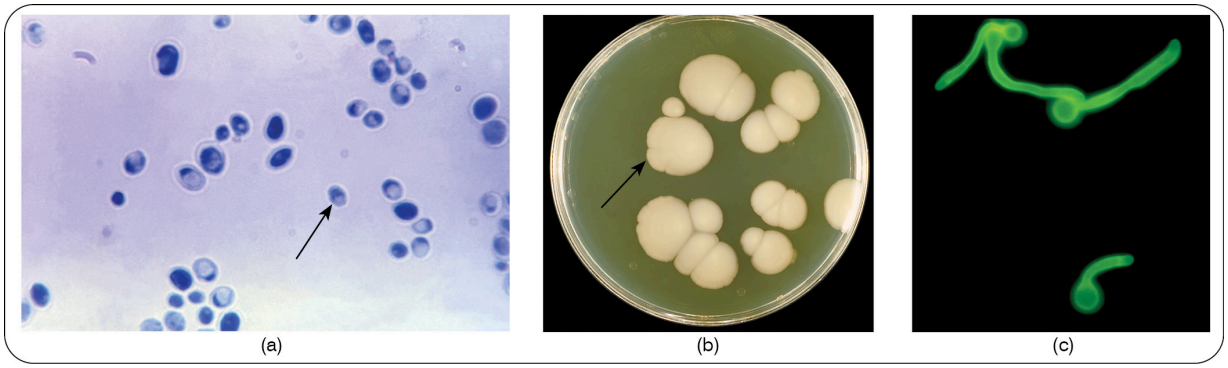


Fig. 57.16 *Candida albicans:* (a) oval budding yeast cells by Gram stain); (b) cream-coloured pasty colonies and (c) germ tubes stained by calcofluor white (*Source:* (a) Public Health Image Library, ID 22294 / Dr. Lucille K. Georg / CDC; (b) Public Health Image Library, ID 3192 / Dr. William Kaplan / CDC and (c) Public Health Image Library, ID 295 / Mercy Hospital, Toledo, OH; Dr. Brian Harrington / CDC)

Treatment

Amphotericin B, 5-fluorocytosine, imidazoles (miconazole, ketoconazole), triazoles (itraconazole, fluconazole, voriconazole) and echinocandins (caspofungin, micafungin) may be used for disseminated candidosis. Some clinical isolates of *C. albicans* are resistant to fluconazole and *C. krusei* to amphotericin.

Prevention of Infection

This is mainly by eliminating possible predisposing factors. All *Candida* strains are susceptible to nystatin but, as it is poorly absorbed from the gut, it is not useful in treating systemic diseases.

CRYPTOCOCCOSIS (TORULOSIS)

Cryptococcosis (torulosis) is a subacute or chronic infection caused by the yeast *Cryptococcus neoformans* and, less frequently, other species.

Distribution

Cryptococcosis occurs worldwide since the fungus is a soil saprophyte and is particularly abundant in the feces of pigeons and other birds. Since cryptococcosis was originally reported from Europe, it was formerly referred to as European blastomycosis. Cryptococcosis has been identified as a common opportunistic infection in AIDS patients.

Clinical Case 2

A 27-year-old woman presented to the emergency department with altered sensorium and neck rigidity. The patient was HIV-seropositive. A lumbar puncture was carried out under aseptic conditions and sent to the laboratory. Gram stain revealed gram-positive, round, budding yeasts (no pseudohyphae). India ink preparation showed capsulated budding yeast cells. Serum cryptococcal latex agglutination test was positive (1:512). The culture on birdseed agar showed black colonies; urease test was positive and culture confirmed the organism as Cryptococcus neoformans. The patient was treated with amphotericin B and flucytosine.

Causative Agent

Cryptococcus neoformans is a round or ovoid budding cell, 4–20 μm in diameter, with a prominent polysaccharide capsule. While *C. neoformans* var. *neoformans* is the principal pathogenic species, another species, *C. neoformans* var. *gatti* is also implicated in human infection. Others include *C. albidus* and *C. laurentii*.

Four serological types of cryptococcal capsular polysaccharide—A, B, C and D—have been identified.

Teleomorphs of the fungus belong to the class Basidiomycetes: *Filobasidiella neoformans* and *F. basiliospora*.

Pathogenesis and Clinical Features

Infection is usually acquired by inhalation but may sometimes be through the skin or mucosa. Most infections are asymptomatic.

Pulmonary cryptococcosis may lead to mild pneumonitis.

Dissemination of infection leads to visceral, cutaneous and meningeal disease. Visceral cryptococcosis may simulate tuberculosis and cancer clinically; bones and joints may be involved. Cutaneous cryptococcosis varies from small ulcers to large granulomata.

Cryptococcal meningitis and meningoencephalitis, the most serious type of cryptococcal infections, can mimic tuberculous or other chronic types of meningitis. Its onset is insidious and the course slow and progressive. It is

often seen in AIDS (*Clinical case 2*).

Laboratory Diagnosis

This is established by direct microscopic examination and by culture.

1. **Microscopy** Microscopic examination of **India ink-stained** CSF and other material from lesions reveals capsulated, budding yeast cells; the capsules are prominent in the India ink preparations (Fig. 57.17a).

2. **Culture** The fungus grows readily on **Sabouraud agar**, forming smooth, mucoid, cream-coloured colonies (Fig. 57.17b). The ability to grow at 37°C and hydrolyse urea differentiates *C. neoformans* var. *neoformans* from non-pathogenic cryptococci.

Pathogenicity of a cryptococcal isolate can be demonstrated by intracerebral or intraperitoneal inoculation into mice, which develop a fatal infection. Capsulated budding yeast cells can be demonstrated in the brain of infected mice.

3. **Antigen detection tests** Demonstration of the capsular antigen by precipitation and latex agglutination can be of value in diagnosing cryptococcal meningitis.

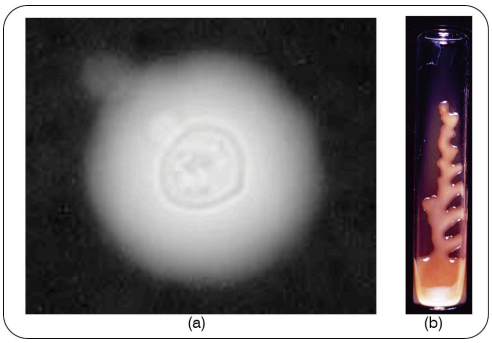


Fig. 57.17 (a) *Cryptococcus neoformans* var. *neoformans*: India ink preparation of spinal fluid showing yeast cells surrounded by a large capsule and (b) mucoid colony (*Source:* (b) Public Health Image Library, ID15677 / CDC)

Treatment

Amphotericin B, 5-fluorocytosine, imidazoles (miconazole, ketoconazole), triazoles (itraconazole, fluconazole, voriconazole) and echinocandins (caspofungin, micafungin) may be used.

PNEUMOCYSTIS JIROVECII

Pneumocystis was, until recently, thought to be a protozoan, but nucleic acid sequencing has conclusively shown that the organism is a fungus related to Ascomycetes. There are two known species: *P. carinii*, commonly found in rats, and *P. jirovecii*, seen in humans. Pneumocystis pneumonia is one of the common opportunistic infections in AIDS.

P. jirovecii has three morphological forms:

1. Trophozoite—thin-walled, irregularly shaped, 1–5 μm in size
2. Precyst—an intermediate stage of the sexual phase, 5–8 μm in size
3. Cyst—thick-walled and spherical, containing up to 8 intracystic bodies and up to 8 μm in size

Pathogenesis and Clinical Features

Infection is transmitted by respiratory droplets and is asymptomatic in immunocompetent individuals. In immunocompromised patients, life-threatening pneumonia develops. X-ray shows interstitial lung infiltrates (Fig. 57.18a).

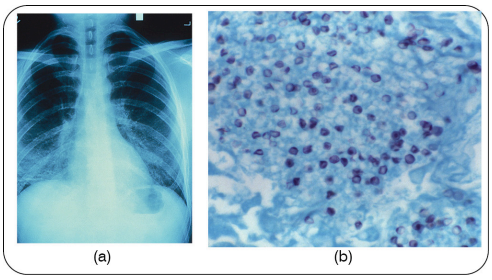


Fig. 57.18 (a) X-ray shows interstitial lung infiltrates and (b) **(GMS)**: *Pneumocystis jirovecii* fungal elements in the alveolar spaces (*Source:* (a) Public Health Image Library, ID 14372 / Jonathan W.M. Gold, MD / CDC and (b) Public Health Image Library, ID 15734 / Francis W. Chandler, DVM, PhD / CDC)

Laboratory Diagnosis

1. **Specimen** Induced sputum, bronchoalveolar lavage or lung biopsy are collected for investigations.

2. **Microscopy** Trophozoites can be demonstrated by Giemsa, toluidine blue, **Grocott's methenamine silver stain** (Fig. 57.18b) and calcofluor white stains. The cyst wall stains black with methenamine silver stain.

3. **Culture** The organism cannot be cultured.

4. **Serology** Complement fixation test with titres of 1:4 or above indicates active disease. Latex agglutination test can also be used.

Treatment

Trimethoprim-sulphamethoxazole (TMP-SMZ) and pentamidine isothionate are the drugs of choice for the treatment of acute cases. TMP-SMZ is also used as prophylaxis in the management of HIV-infected individuals.

SPECIFIC FUNGAL INFECTIONS

OTOMYCOSIS

Otomycosis is a fungal infection of the external ear. It is a very common disease and is usually caused by species of *Aspergillus* (*A. niger*, *A. fumigatus*) and *Penicillium.* The symptoms are itching, pain and deafness. Secondary bacterial infection, commonly due to *Pseudomonas* species and *Proteus* species, causes suppuration. Diagnosis can be made by the demonstration of the fungi in scrapings and by culture.

OCULOMYCOSIS (KERATOMYCOSIS, FUNGAL KERATITIS, MYCOTIC KERATITIS)

Mycotic keratitis is an invasive infection of the cornea, usually following corneal trauma.

Causative Agents

Many saprophytic fungi can cause ocular infection. *Aspergillus* species (*A. fumigatus, A. flavus* and *A. niger*), *Fusarium, Curvularia, Alternaria, Acremonium* and *Candida albicans* are most often responsible.

Pathogenesis and Clinical Features

Corneal injury and bacterial infection are predisposing factors for fungal keratitis. The widespread use of corticosteroids in ophthalmology has resulted in the increased incidence of keratomycosis.

Fungal spores colonise the injured tissue and initiate an inflammatory reaction, leading to hypopyon ulcer and endophthalmitis.

Laboratory Diagnosis

Specimen Corneal scrapings are collected under slit lamp examination (to visualise the ulcer margin and extent). The material is used for microscopy (Fig. 57.19) and culture.

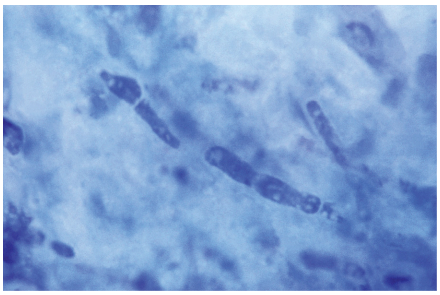


Fig. 57.19 Corneal scrapings **from a patient diagnosed with a case of mycotic keratitis showing broad septate filaments (***Source:* **Public Health Image Library, ID 15468 / Dr. Lucille K. Georg / CDC)**

Treatment

Local application of amphotericin B, nystatin and pimaricin (natamycin) may be useful.

MYCOTIC POISONING

Many fungi form poisonous substances. Mycotic poisoning is of two types: **mycetism,** in which the ingestion of the fungus causes toxic effects, and **mycotoxicosis,** in which fungal toxins contaminate food, which on ingestion, cause infection.

Mycetism

This has been known from ancient times, several varieties of poisonous mushrooms having been identified as inedible. Mycetism may cause gastrointestinal disease, dermatitis or death. Some examples are:

- *Claviceps* species—ergot poisoning
- *Coprine* species—coprine poisoning
- *Inocybe* species—muscarine poisoning

The hallucinogenic agents (d-lysergic acid, psilocybin) produced by the *Psilocybe* species and other fungi have attracted much attention in recent

years.

Mycotoxicoses

These are diseases caused by the toxic metabolic products released by fungi and result from the ingestion of food contaminated with mycotoxins (fungal toxins). Examples of this are aflatoxicosis due to the consumption of grains containing aflatoxins secreted by *Aspergillus flavus* which contaminates groundnuts, corn and peas and ergotism after the ingestion of rye contaminated with *Claviceps purpurea.* Here, the fungus does not necessarily have to be present in the tissues to exert its pathogenic effect since its toxic metabolites are present; moreover, there is no invasion of tissues by the fungus. Mycotoxins are natural products produced by fungi and found in some articles of food (Table 57.1).

Table 57.1 Important mycotoxins and their sources

Mycotoxin	Fungus	Contaminated food item
Aflatoxin	*Aspergillus* sp.	Groundnuts, corn and peas
Fumonisin	*Fusarium*	Maize
Ochratoxin	*Aspergillus* and *Penicillium*	Cereals and bread
Ergotoxin	*Claviceps purpurea*	Rye
Zearalenone	*Fusarium graminearum*	Grains
Trichothecenes	Various species of *Trichoderma*, *Trichothecium,* and *Myrothecium*	Livestock feeds or foodstuff

Aflatoxin The best known mycotoxin is that of the aflatoxin group: *Aspergillus flavus* secretes aflatoxin B1 while *Aspergillus parasiticus* secretes aflatoxins B1, B2, G1 and G2, among others. Aflatoxins are frequently present in mouldy foods, particularly in groundnuts, corn and peas. These toxins are highly toxic to animals and birds, as well as to human beings. Aflatoxins are known to cause hepatomas in ducklings and rats, and their possible carcinogenic effect in human beings has caused great concern. There have been several reports of aflatoxicosis from India, involving human beings and animals.

Ergot alkaloids Ergotoxicosis (ergotism) is due to the toxic alkaloids produced by the fungus *Claviceps purpurea*, while growing on the fruiting

heads of rye. Trichothecenes are toxins produced by certain species of *Fusarium*. Zearalenone is a toxin produced by *Fusarium graminearum*; animals that consume grains contaminated with this toxin may develop symptoms and signs mimicking an estrogenic disorder.

Key points

- Histoplasmosis is caused by *Histoplasma capsulatum* var. *capsulatum* (classical form) and *Histoplasma capsulatum* var. *duboisii* (African histoplasmosis). The fungi are found in soil (bird droppings), and enter the body by inhalation.
    - African histoplasmosis frequently spreads to the skin and bones. Classical histoplasmosis manifests as a pulmonary infection (usually self-limiting).
- In tissues, small intra- and extracellular yeast cells (classical form) or large, thick-walled yeast cells and giant cells (African form) are diagnostic; in culture, tuberculate spores are characteristic.
    - Serological tests and skin test (histoplasmin) may aid diagnosis.
- Blastomycosis is caused by *Blastomyces dermatitidis* (thermally dimorphic fungus). It grows as a yeast at 37°C and as a filamentous fungus at room temperature.
    - Following inhalation of spores, pulmonary and systemic granulomatous lesions result.
    - The diagnostic form is a large yeast with a single broad-based bud.
- Paracoccidioidomycosis is a chronic granulomatous disease of the skin, mucosa, lymph nodes and internal organs, caused by *Paracoccidioides brasiliensis*, a thermally dimorphic fungus.
    - The yeast phase (in tissues and in cultures at 37°C): large, globose or oval cells with multiple buds encircling the mother cell—mariner's wheel or pilot's wheel appearance.
    - The mycelial phase: found in nature; also isolated in culture at 25–30°C after 2–3 weeks incubation.
- Coccidioidomycosis is caused by *Coccidioides immitis*, a thermally dimorphic fungus. The hyphal phase is found in the soil of southwestern USA; conidia (arthrospores) are inhaled with dust.
    - Most cases are asymptomatic; some progress to self-limiting pneumonitis.
    - Cultures are dangerous to laboratory workers due to the release of arthrospores. The diagnostic tissue form is the spherule.
- Aspergillosis is an opportunistic mycosis caused by hyaline filamentous fungi of the genus *Aspergillus*. The key species, *A. fumigatus*, *A. flavus* and *A. niger*, produce a spectrum of diseases ranging from superficial (otitis externa leading to otomycosis) to invasive lesions affecting all tissues.
    - Diagnosis is by demonstration of septate hyaline hyphae in tissue material and isolation of the species in culture.

- Zygomycosis is an opportunistic mycosis by fungi of the class Zygomycetes (mainly *Mucor* and *Rhizopus*). Fungi appear in biopsy material as broad-branched hyphae with no cross-walls; surrounding tissue necrosis, thrombosis and hemorrhage are prominent. They grow as moulds on ordinary culture media.
    - Rhino-orbito-cerebral zygomycosis runs a rapid course in poorly-controlled diabetics.
- Candidosis is an opportunistic mycosis caused by yeast-like fungi of the genus *Candida*; *C. albicans* is the main pathogen. Lesions (thrush) may occur on oral and vaginal mucous membranes of otherwise healthy individuals. In immunocompromised hosts (including diabetics), lesions may be widespread.
    - *Candida* species grow on ordinary culture media at 37°C or 25°C yielding smooth or butyrous colonies.
    - *Candida albicans* forms chlamydospores on cornmeal agar and germ tubes in human serum; in tissues and exudates, yeasts and pseudohyphae are found.
- Cryptococcosis is an opportunistic mycosis caused by yeast fungi of the genus *Cryptococcus*; *C. neoformans*, the principal pathogen, is heavily encapsulated, reproduces by budding and is widely distributed in nature (particularly soil and pigeon droppings).
- *P. jirovecii,* commonly associated with pneumonia in AIDS patients, has the following features:
    - It has three morphological forms: trophozoite, precyst and cyst.
    - Infection occurs by respiratory droplets and clinically, may vary from asymptomatic to life-threatening pneumonia.
    - The organism cannot be cultured.
    - The treatment of choice is trimethoprim-sulphamethoxazole (TMP-SMX).
- Otomycosis is a fungal infection of the external ear, commonly caused by *Aspergillus* and *Penicillium*.
- Oculomycosis/keratomycosis is a fungal infection of the cornea.
    - The common causative agents include *Aspergillus, Fusarium, Curvularia* and *Alternaria*.
    - Diagnosis is made by microscopy and culture of corneal scrapings.
- Mycotic poisoning is of two types: mycetism in which the ingestion of a fungus causes toxic effects and mycotoxicosis in which fungal toxins contaminate some articles of food.

Questions

Essays

1. Name the fungi causing systemic infection. Describe the pathogenesis and laboratory diagnosis of *H. capsulatum*.
2. Name the fungi causing opportunistic fungal infections. Describe the pathogenesis and laboratory diagnosis of any one.

Short Answers

1. Dimorphic fungi

2. Candidosis
3. *Cryptococcus neoformans*
4. *Histoplasma capsulatum*
5. Opportunistic fungi
6. Aspergillosis
7. Mucormycosis
8. Otomycosis
9. Oculomycosis
10. Penicillosis
11. *Penicillium marneffei*
12. *Pneumocystis jirovecii*
13. Mycotoxins
14. Mycetism

Part V: Medical Mycology

Chapter 58

Laboratory Diagnosis of Fungal Infections

Chapter navigation

- Specimens
- Microscopy
- Culture
- Media for fungus culture
  - Sabouraud dextrose agar
  - Morphological identification
- Preparation of slides
- Tests for identification
- Testing antifungal agents for treatment of fungal infections

INTRODUCTION

Medical advances have increased the survival rate of patients with life-threatening infections. Increasing incidences of immunocompromised individuals and chronic debilitating diseases like tuberculosis and diabetes have contributed to the increasing incidence of fungal infections. Clinicians have to depend on diagnostic laboratories in health care settings to identify the causative agents and sometimes do a susceptibility test against antifungal agents as resistance to antifungal agents is increasing. A detailed clinical examination and recording of the patient's occupational history and geographic location are important in diagnosing a fungal infection. Demonstrating the fungal elements in the clinical specimen is one of the important methods to correlate the agent with the infection as many of the fungi may be found in the environment.

As many fungi sporulate and can be potentially pathogenic, it has been recommended that all laboratory procedures be carried out in

biosafety cabinets (Class II B with negative pressure and HEPA filter).

The collection of the appropriate specimen prior to starting any antifungal therapy is also necessary for isolating the causative agent. Like most infectious agents, the three cardinal laboratory procedures are:

1. Direct demonstration of the fungal elements in the clinical material by microscopy (as a rapid method to detect fungal elements)
2. Isolation of the fungus by culture (to isolate for morphological identification, and susceptibility testing for some of them)
3. Immunological evidence of infection—detection of antigens (or antibodies, which are not usually recommended) by immunological tests

Other tests may be:

- Skin tests (for hypersensitivity)
- Molecular methods—to identify rare species

SPECIMEN

The specimen to be collected depends on the site of involvement (Table 58.1).

Table 58.1 Specimens from different types of fungal infections

Site of infection	Clinical material
Superficial	Skin scrapings, hair and nail clippings
Subcutaneous	Tissue biopsy
Mycetoma	Granules from the sinus or tissues from the sinus tract
Deep and opportunistic infections	Blood, CSF, pus discharge, sputum, pleural and peritoneal fluid, other aspirates or any other material from the site of infection

LABORATORY METHODS OF ISOLATION AND IDENTIFICATION

1. Microscopy

Fungal structures can be detected in clinical specimens by direct microscopic examination of the material from the lesion and by morphological study of fungal isolates. The following methods are used to detect these fungal elements:

i) **Potassium hydroxide (KOH) preparation—**tissue specimens such as skin scrapings are generally examined as wet mounts after treatment with 10% potassium hydroxide (20% for nail clippings). KOH digests keratin, cells and other tissue materials, enabling the fungal elements to be seen clearly.

ii) **Calcofluor white (CFW)** is a sensitive staining procedure that provides good visualisation of the morphology of the fungus.

iii) **Gram stain** is useful in identifying yeast and yeast-like fungi.

iv) **India ink** is used for negative staining of capsulated yeast, e.g., *Cryptococcus*.

v) **Methenamine silver stain** and **periodic acid–Schiff (PAS)** are valuable methods used for demonstrating fungi in tissues.

vi) **Lactophenol cotton blue (LCB)** is used for the microscopic study of fungus colonies that are teased on to a slide and mounted.

2. Culture

Fungi are slow-growing and, therefore, need to be cultivated in media that can support the growth for a period of two to three weeks without drying. The points in the following box are considered when attempting to culture fungi in the laboratory.

Requisites for fungal culture

- Most fungi are grown at a lower pH (5.4–6) in media similar to those used for bacteria.
- All fungi require oxygen for growth.
- Fungi grow better at a temperature of 25–30°C (exceptions are those that cause deep mycoses or dimorphic fungi that grow well at 37°C and *Aspergillus fumigatus* that can grow even at 50°C). They are usually incubated in BOD (biochemical oxygen demand) incubators.

Media for fungus culture

Sabouraud dextrose agar This culture medium is most commonly used. It permits the growth of yeasts and most filamentous fungi. It has a high concentration of either glucose or maltose and also contains mycological

peptone. The medium has a low pH (5.4), which inhibits the growth of most bacteria. Antibacterial agents (chloramphenicol, 40 mg or gentamicin, 50 mg per litre of medium) can also be added to inhibit bacteria that may be present in the specimen. Cycloheximide (Actidione) incorporated in the medium inhibits many contaminant moulds.

Some of the other media used are:

- Czapek–Dox medium
- Dermatophyte test medium
- Birdseed agar
- Cornmeal agar
- Chromogenic agar (CHROM agar), which produces different coloured colonies by different species of candida (Fig. 58.1)
- Blood agar, brain heart infusion agar or brain heart infusion broth

Cultures are routinely incubated in parallel at room temperature (22°C) for 3–4 weeks and at 37°C for one to two weeks. Identification is based on the morphology of the fungus and its colony. Diagnostic mycology rests largely on a detailed study of the morphological evolution of the isolate.

Fig. 58.1 CHROM agar showing different coloured colonies produced by different species of candida (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

3. Morphological Identification

Growth characteristics useful for identification

- **Rate of growth** at 37°C or room temperature (25°C) (yeasts and saprophytic moulds grow within 48 to 72 hours, whereas pathogenic fungi such as paracoccidioides, which are obligate pathogens grow

slowly)
- **Colour and morphology** (pale, creamy, pigmented) of the colony and its **texture** (velvety, granular or cottony)
- **Pigmentation** on the obverse (front surface) or reverse side (reverse vs obverse) and whether **diffusible or non-diffusible**
- **Morphology of various structures**—hyphae (diameter, type, septa), conidiogenous cells, fruiting bodies, conidia (spores), manner of their attachment to the conidiogenous cell, and other special structures

Preparation of slides

The following are the methods of preparing slides for studying morphological features.

i) ***Teased mounts*** This is the easiest to prepare. A bit of the fungal colony is taken and placed in a drop of lactophenol cotton blue on a clean, grease-free slide. The mount is then '**teased out**' using a bent wire. It is then covered with a coverslip and viewed under the microscope. The disadvantage of this method is that it breaks the hyphae and scatters the conidia, making the fungus difficult to identify.

ii) ***Slide culture*** This has the advantage of preserving the 'in situ' appearance of the fungal structures, without any breakage of hyphae or attachment of conidia to the conidiogenous cell.

A sterile microscopic slide is placed on a bent glass rod in a petri dish. A 1 cm square block of SDA is placed on the slide. The fungal strain to be identified is inoculated at the four sides of the agar block. The inoculated block is covered with a sterile coverslip and incubated at 25°C. After 48 hours, growth appears; then, a drop of LCB is placed on a fresh slide and the coverslip is transferred from the block of SDA onto this slide. The preparation is viewed under the microscope. The undisturbed morphology of the fungus is observed (Fig. 58.2).

Fig. 58.2 Slide culture showing agar blocks with fungal culture (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

iii) ***Cellophane tape mount*** This is a compromise, being technically easier to perform while at the same time, allowing the 'in situ' appearance to be viewed under the microscope; however, the fungal culture may subsequently become contaminated due to contact with the unsterile cellophane tape.

4. Tests for Identification

i) Yeasts can be identified by **carbohydrate fermentation and assimilation** tests.

- **Fermentation test** is done in a medium consisting of 1% yeast extract and 2% test carbohydrate in distilled water. It is dispensed in fermentation tubes containing Durham tubes. Carbohydrate fermentation occurs within 7 days. The ability of yeasts to ferment carbohydrates is determined strictly on the basis of gas production from the substrate (Fig. 58.3).

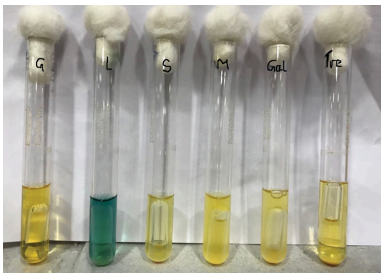


Fig. 58.3 Carbohydrate fermentation tests showing production of gas (*Source:* Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry)

- **Assimilation test** determines the ability of a yeast isolate to use a particular carbohydrate substrate as its sole carbon source for metabolic process in a medium. The medium used is a yeast nitrogen base onto which the yeast is inoculated as a carpet. Filter paper discs containing 2% test carbohydrate are placed on the plate. Colonies of the yeast grow around the disc of the carbohydrate it assimilates, enabling it to grow.

Automated systems like API, VITEK and MicroScan can be used to rapidly identify candida species.

ii) **Serological tests** are done to demonstrate either antigen or antibody in serum or body fluids:

- **Antibody detection**
  - Immunodiffusion tests—antibodies to *Coccidioides* can be detected by immunodiffusion.
  - Complement fixation test—Antibodies to some invasive fungal infections become detectable in 2 to 3 weeks, e.g., antibodies to histoplasmin antigen.
  - Immunoassays are highly sensitive in detecting antibodies to soluble antigens of some fungi, e.g., *Histoplasma capsulatum*.
- **Antigen detection**
  - Indirect fluorescent antibody tests in tissue sections.
  - Latex agglutination for cryptococcal capsular antigen, *Candida* and *Aspergillus*.

iii) **Immunohistochemistry** is useful for a number of important invasive pathogens in tissue sections.

iv) **Skin test** is used to detect delayed-type hypersensitivity (DTH) for pathogens like *Histoplasma* or *Coccidioides*.

v) **Newer rapid diagnostic tests** to identify newer fungal species that cause human infections are:

- Nucleic acid hybridisation
- Polymerase chain reaction

vi) **Antifungal susceptibility tests** are not undertaken routinely due to cumbersome and time-consuming methods. However, susceptibility testing is done for some yeasts and moulds by two methods:

a. **Disc diffusion tests** are inexpensive and easy to set up, and provide a screening test. The disc diffusion method mainly tests azoles (e.g., fluconazole) and echinocandins (e.g., caspofungin)

for *Candida* spp. and some dermatophytes. Media used is Mueller-Hinton agar supplemented with 2% glucose.

b. **Microbroth dilution methods** are used for yeasts and some filamentous fungi, against antifungal agents like amphotericin, fluconazole and echinocandins. Based on standardised breakpoints, the susceptibility can be determined.

Semi-automated and automated (e.g., VITEK) methods are now available to test for candida.

TREATMENT

Antifungal Agents

Medical therapy of mycotic infections involves the use of compounds belonging to different groups: polyenes, pyrimidines, azoles, grisans and echinocandins (Table 58.2). Some medications are used only topically for application on the skin or mucous membranes (pimaricin for eye or skin infections), some only orally (griseofulvin) while others (amphotericin B, some azoles) can be administered by a variety of routes.

Table 58.2 Antifungal agents used to treat fungal infections

Class	Compounds	Mechanism	Uses
Polyene	Amphotericin B Nystatin, Pimaricin	Binds to sterols causing perturbations in cell membrane	Systemic disease, skin infection and eye infection
Imidazole and triazole (synthetic antifungal agents: azoles)	Itraconazole Voriconazole Ketoconazole	Inhibits ergosterol biosynthesis	Topical disease (ringworm), systemic disease
Pyrimidine	5-Fluorocytosine (5-FC)	Inhibits DNA and RNA synthesis	Used in association with amphotericin B
Grisans	Griseofulvin	Inhibits microtubule assembly	Dermatophytoses
Echinocandins	Caspofungin Micafungin	Inhibits beta-glucan synthesis in cell wall	Systemic diseases due to *Candida* and *Cryptococcus*

Key points

- Fungi are eukaryotic Protista that possess a rigid cell wall containing chitin, mannan and other polysaccharides. They have true nuclei with nuclear membrane and paired chromosomes.
- Medically important fungi are classified based on their morphology as yeasts, yeast-like fungi, moulds (filamentous fungi) and thermally dimorphic fungi, while from the standpoint of disease-causing potential in humans, fungi are regarded as primary pathogens and opportunist pathogens.
- Specimens for diagnosis are usually collected from the affected site (also from blood in disseminated infections). Specimens are examined by direct microscopy or used for culture (Sabouraud dextrose agar). New diagnostic methods are increasingly being used.
- Antifungal susceptibility tests can be done by disc diffusion, microbroth dilution or semi-automated and automated methods.
- Antifungal agents include polyenes, azoles, pyrimidines, grisans and echinocandins.

Questions

Essay

1. Describe the laboratory diagnosis of fungal infections.

Short Answers

1. Sabouraud dextrose agar
2. Culture media used in mycology
3. Slide culture

Part VI:
Clinical Microbiology as Applied to Infectious Diseases

Part VI: Clinical Microbiology as Applied to Infectious Diseases

Chapter 59

Diagnostic Microbiology

Chapter navigation

- Introduction to infectious diseases
- Principles of laboratory diagnosis of infectious diseases

INTRODUCTION

Infectious diseases remain one of the most common causes of morbidity and mortality world over despite advances in medical sciences. They are responsible for the loss of economy globally, evident more in developing countries and among underprivileged populations. These diseases are a global concern because they can be transmitted across countries and continents. Fortunately, they are preventable with improved standards of living, immunisation and upgradation of the public health system. Factors that influence infectious diseases and their changing epidemiology are as follows:

- Emergence of new infections such as Ebola, human metapneumovirus, zoonotic flu virus (H1N1), SARS, MERS-CoV and Covid-19
- Re-emergence of old infections like chikungunya

Other factors that influence infectious diseases include:

- Geography
- Environment
- Climate
- Migration of population

Interaction of host, parasite and environment are the key to the manifestation of infection. This has been explained in Chapter 7.

Clinical manifestations of infectious diseases are varied, from acute life-threatening infection (Ebola) to chronic, indolent infection for prolonged periods of time (tuberculosis). They may be mild (common cold), subclinical or acute florid infection (exanthems).

Infections may present as solitary episodes (sepsis) or as outbreaks in the form of epidemics (dengue, chikungunya, Nipah, Ebola) and pandemics (influenza, COVID-19).

The knowledge of the natural history of the infection, its geographic preponderance and clinical presentation is essential to its diagnosis.

A detailed history of travel, occupation and behaviour, besides thorough physical examination are key to the diagnosis of infectious diseases.

Laboratory investigations are aimed at establishing an etiological diagnosis. Hence they must be carefully considered to get the best result in the shortest possible time and must be cost-effective.

Laboratory investigations use scientific methods for the rapid and accurate diagnosis of infectious diseases. Once the causative agent of a particular infection has been identified, appropriate and rational therapy can be instituted. With rapidly improving diagnostic techniques that give reliable and timely results, there is a greater need for laboratory procedures to be standardised and to adhere to good practices. The diagnosis of infectious diseases relies upon the isolation and identification of the causative agent or the demonstration of the specific antigen in the infected tissue. Recent or past infections can be diagnosed by the demonstration of antibodies in the serum of the patient. Detection of IgM in the serum denotes recent or ongoing infection, whereas the demonstration of IgG reflects a past infection. Before requesting laboratory investigations, the clinician should have arrived at a provisional diagnosis or the probable cause of infection based on an accurate and detailed history related to the illness and careful physical examination.

Appropriate clinical specimens should be collected from sites most likely to yield the microorganism. Once the sample has been received by the laboratory, tests are performed using standard operating procedures. The outcome of the test results are then documented and sent to the treating clinician for appropriate treatment. A communication between the clinician

and laboratory personnel is essential to get the best result from the laboratory. It is, therefore, prudent to consult the laboratory on the tests to be performed, the types of specimens to be collected for the tests, and the mode of transport of these specimens. For this reason, it is important to have an understanding of the principles behind the collection of appropriate specimens for microbiological examinations.

The details of laboratory diagnosis have been discussed in individual chapters. This chapter gives an overview of the laboratory process and quality control.

STEPS IN THE LABORATORY DIAGNOSTIC PROCESS

- Pre-test process (pre-analytical phase), involving specimen collection and transport
- Test procedures and processing of the specimen in the laboratory (analytical phase)
- Post-test process (post-analytical phase), involving reporting to the clinician and archiving

SPECIMEN COLLECTION AND TRANSPORT (PRE-TEST)

The outcome of a laboratory test depends, to a large extent, on the appropriateness of the specimen in terms of selection, quality, quantity, time (prior to antibiotics or other treatment) and method of collection.

Precautions for sample collection

- If culture and isolation of the pathogen is required, asepsis during collection of the sample is essential to avoid contamination by skin flora or environmental organisms. The container in which the sample is to be collected must be sterile and has to be adequate to contain the material. Samples for anaerobic culture require an anaerobic container or transport media.
- The specimen should be transported to the laboratory without undue delay. If this is not possible, it should be stored at an appropriate temperature for a specified time until transport.
- For the culture of viruses, specimens need to be transported in viral transport media. Viral transport media prevent the drying of the specimen, maintain viability of the viruses and prevent overgrowth by contaminating flora. The specimen should be held at 4°C during transport for most viral isolation studies.

Sites of Sample Collection

The sample should be representative of the infection, for example, sputum and not saliva should be collected to diagnose pulmonary tuberculosis. The type of specimen to be examined is determined by the clinical presentation of the infection (Table 59.1).

Table 59.1 Sites of infection and corresponding samples for microbiological tests

Site	Sample
Skin	Scrapings, swabs and biopsies
Pus	Aspirates and drained fluid
Nail and hair	Clippings
Conjunctiva	Swabs
Ear	Swabs
Respiratory tract: • Upper tract • Lower tract	Throat swabs, nasopharyngeal / nasal swabs / nasal wash / sinus aspirates Sputum (not saliva), bronchoalveolar lavage
Gastrointestinal tract	Stool samples, vomitus, endoscopic biopsies
Genito-urinary tract	Vaginal and cervical swabs, urethral discharge
Urinary tract	Urine, suprapubic aspirate
Central nervous system	Cerebrospinal fluid (CSF)
Bloodstream infections	Blood, bone marrow
Intraoperative samples from internal organs	Clotted blood (serum) or plasma for immunological tests to demonstrate antigens or antibodies

PROCESSING OF SAMPLES IN THE LABORATORY (TEST PROCEDURE)

Once the appropriate specimens have been collected, they are processed as speedily as possible to ensure that the organisms do not die before being transferred to the culture media, and that the reports are available as early as possible.

The specimens processed in the microbiology laboratory are potentially hazardous since the etiological agents are unknown at the time of processing. For this reason, it is advisable to process all biological samples in a biosafety cabinet. A sample could be of moderate or high individual risk but low risk to community (biosafety level-1 and 2). On the other hand,

organisms that have high individual risk and pose high risk to community as well, need to be processed in containment laboratories (level-3) with high levels of biosafety.

Based on the category of the infectious agent to be processed, biosafety cabinets are designed to control air circulation by HEPA (high-efficiency particulate air) filters within the cabinet. In a diagnostic microbiology laboratory, Class I and Class II cabinets are used.

Gross Examination of the Specimen

This may help to correlate with the clinical condition, e.g., bloodstained CSF may suggest traumatic tap or subarachnoid bleed due to anthrax meningoencephalitis.

Microscopy

This is the first step in the laboratory procedure and often helps in giving a rapid diagnosis. Generally, a portion of the specimen is placed on slides, stained appropriately and then examined under the microscope. If bacteria and fungi are swiftly detected, specific therapy can be started at once.

Staining

The Gram stain is the most common stain used in diagnostic microbiology. It helps in provisionally identifying the organism as gram-positive or gram-negative. Based on this empiric evidence, antibiotics may be started. The Gram stain is found most useful for the rapid diagnosis of pyogenic meningitis. Sterile body fluids and aspirates can also be stained for rapid diagnosis. This can also be used to detect inflammatory cells in the specimen, thus providing additional information.

Some of the preliminary staining methods are:

- Acid-fast staining (Ziehl–Neelsen and Kinyoun stain)
- Fluorochrome staining (calcofluor white or acridine orange)
- For the detection of fungal elements, 10–20% KOH is added to the specimen before examining it under the microscope or by staining with calcofluor white

Culture and Identification

Based on the outcome of the preliminary microscopy result, the specific media and method of incubation (at 37°C, ambient temperature or in CO_2) can be selected for the culture of the specimen. Generally, after overnight incubation, the colonies on culture plates are processed for identification of the organisms.

Some of the common tests used to identify bacteria are the hanging drop test for motility; breakdown of carbohydrates, glucose, sucrose and lactose using composite media like triple sugar iron agar (TSI); tests for the production of gas and hydrogen sulphide; utilisation of citrate as the sole source of carbon and tests for the production of enzymes like oxidase and urease. These tests identify most of the common gram-negative pathogenic bacteria. Final identification of some bacteria like *Salmonella* and *Shigella* is based on serotyping with specific antisera. Similarly, tests like coagulase, hippurate hydrolysis and bacitracin susceptibility are performed for gram-positive cocci. The organisms are simultaneously subjected to antibiotic susceptibility test by Kirby–Bauer disc diffusion method. Some laboratories identify the microorganisms by automated VITEK/MicroScan systems (discussed in detail in the respective chapters).

Rapid Diagnostic Methods

To decrease the **turnaround time or TAT** (the time from the collection of sample to the reporting of the results to the physician), several automated and semi-automated rapid diagnostic methods such as automated culture and identification systems (MALDI-ToF, VITEK, MicroScan, etc.) are used (discussed in Chapter 6).

Rapid Identification of Infectious Diseases

Some life-threatening infections like acute pyogenic meningitis, peritonitis, sepsis and some viral infections need to be diagnosed rapidly to start treatment immediately.

Immunological tests for antigen detection

- Latex agglutination test for detecting *Haemophilus*, *Pneumococcus*, *Meningococcus*, Group B *Streptococcus* or *E. coli* antigen from the CSF in acute pyogenic meningitis
- Cryptococcal antigen in meningitis in immunocompromised individuals
- NS1 antigen from the serum of patients with dengue
- Enzyme immunoassay (EIA), including enzyme-linked immunosorbent assays (ELISA) for the detection of antigens (e.g., scrub typhus)

Molecular methods

This includes the detection of DNA or RNA by various molecular methods like conventional PCR, real-time PCR, multiplex PCR, etc., (Chapter 66) and the very recent innovation, the BioFire FilmArray, which is a multiplex PCR system that integrates sample preparation, amplification, detection and analysis.

Serological tests for the detection of IgM and IgG

Etiological agents cannot always be isolated or detected in the laboratory by conventional or automated culture systems. This is overcome by detecting specific antibodies in the patient's serum.

- ***Detection of IgM by ELISA*** This denotes the early phase of the infection. The antibodies disappear in the later phase. This test is done to detect dengue virus infection.
- ***Detection of IgG*** The presence of IgG denotes exposure to the infection (*Brucella* agglutination test).
- ***Western blot*** This test is done to detect antibodies against specific antigens of a microorganism, for example, antibodies against an array of antigens in an HIV-positive person's serum confirms the infection.

Isolation of the virus requires cell culture systems, which are not available in most clinical diagnostic laboratories. Hence most viral infections are diagnosed by the detection of either viral antigens (HIV) or antibodies (IgM or IgG—arbovirus infections) or by detecting the viral RNA or DNA with

the help of molecular detection methods.

REPORTING OF RESULTS (POST-TEST)

This is a crucial step in the laboratory process. The results of the tests must be conveyed to the treating physician who has requested the investigation. They must be conveyed in standard reporting formats in such a way that the physician or patient is able to get accurate and reliable results, which are clear and easy to understand. A wrong report or an incomplete one might put the patient in danger of receiving the wrong treatment or inadequate management, for example, reporting an HIV-reactive sample as non-reactive or vice versa.

QUALITY CONTROL IN A CLINICAL MICROBIOLOGY LABORATORY

Clinical microbiology laboratories play an important role in ensuring the quality of care. This is ensured by adhering to quality control in all laboratory procedures. In clinical microbiology laboratories, a large number of procedures are used to generate test results. These tests include direct smears such as the Gram stain and the use of different aerobic and anaerobic media, including fungal and mycobacterial media for culture of the suspected pathogens. Several tests for the identification of these pathogens such as biochemical tests, enzymatic reactions and, most importantly, antibiotic sensitivity tests are routinely performed. Immunological tests are done to detect antigens or antibodies from the blood/serum or other specimens from patients. Each individual test and procedure, each type of medium used and even the reporting of results must adhere to prescribed standards. The quality of the procedures must be regularly checked by **quality control (QC) measures**.

All procedures need to undergo quality checks to give reliable results which will ultimately benefit the patient. These procedures are tested against standard controls whose range of activity is known. When tested in laboratory conditions, they must give predetermined results. These checks have to be performed periodically to ensure the quality of the laboratory procedure.

The frequency and type of QC varies according to the test, medium or reagent. For some tests like coagulase or catalase, QC must be performed along with every test. For other tests, QC must be performed only when a new lot number is to be used, for example, when a new lot of antibiotic discs are used. Automated systems in the laboratory, like the ELISA or blood culture systems, require the calibration of the instruments to give reliable results.

Clinicians and infectious disease specialists need to be aware of the quality control systems used in microbiology laboratories. Such awareness helps in the understanding of the laboratory process, right from specimen collection to the receipt of test results.

Laboratory accreditation is a process by which an external agency (a regulatory body) assesses the laboratory practices by checking the processes and procedures and certifies specific tests, to see if they meet the standards of the International Organization for Standardization (ISO). In India, the **National Accreditation Board for Testing and Calibration Laboratories (NABL)** provides certification for tests which have been assessed by a team of assessors and which fulfil the standard quality criteria set down by the ISO. This certification is valid for a specified period of time and pertains only to the tests assessed. NABL accreditation of laboratories is gradually becoming mandatory for laboratories to meet the quality criteria for patient care.

Key points

- Diagnosis of infectious diseases relies upon the isolation and identification of the causative agent or demonstration of the specific antigen in infected tissues.
- Another evidence of recent or past infection is the demonstration of antibodies. IgM denotes recent or ongoing infection, whereas demonstration of IgG reflects past infection.
- The laboratory report is only as good as the specimen that is sent.
- The precautions to be followed while collecting specimens include the following:
    - The process of laboratory investigation should not cause harm to the patient.
    - The specimen should be collected in an aseptic manner.
    - It should be collected before starting antimicrobial therapy.
    - The swab or container used to collect the specimen should be sterile.
- General procedures followed in the laboratory include:

- Microscopic examination of the sample smeared and stained on a slide by Gram, acid-fast or other specific stains
- Culture on a battery of solid (basal/differential, enriched or liquid) media
- Processing of culture isolates for further characterisation and identification
- Antibiotic susceptibility test
- Generation of final report for the clinician or patient
- Detection of antigens or antibodies by immunological/serological tests
- Quality control in all test procedures must be adhered to for reliable reports
- Accreditation for tests done by clinical laboratories is based on adherence to ISO standards, which is certified by a regulatory body. In India, it is the NABL that accredits test procedures done by clinical microbiology laboratories.

Questions

Essay

1. Outline the procedures adopted in the laboratory to isolate and identify pathogens.

Short Answers

1. Common samples received in the microbiology laboratory
2. Precautions required for sample collection

Part VI: Clinical Microbiology as Applied to Infectious Diseases

Chapter 60

Systemic Infections and Their Laboratory Diagnosis

Chapter navigation

- CVS and bloodstream infections
- Gastrointestinal and hepatobiliary infections
- Musculoskeletal, skin and soft tissue infections
- Central nervous system infections
- Respiratory tract infections
- Genitourinary and sexually transmitted infections
- Vector-borne and zoonotic infections
- Miscellaneous (eye infections, ear infections and congenital infections)

This chapter deals with the application of the science of basic and clinical microbiology to infectious diseases. It describes infectious diseases commonly affecting various systems of the human body. The infections are dealt with under the following heads: pathogenesis and clinical characteristics, etiology with characteristics of the organisms and laboratory diagnosis. Details of pathogenesis, epidemiology, treatment and prevention are dealt with under individual organisms.

Certain infectious diseases cause epidemics and pandemics, affect large populations and are associated with high morbidity and mortality. Some of these systemic infections are also dealt with in this chapter. To prevent and control some of these infections, the government has launched several national control programmes.

CVS AND BLOODSTREAM INFECTIONS

CARDIAC INFECTIONS

Infections of the **cardiovascular system** involving the heart and the blood vessels can be life-threatening unless diagnosed and treated promptly. They are broadly classified as those involving the heart and those involving the blood vessels.

The infections involving the heart include:

- Infective endocarditis
- Myocarditis
- Pericarditis

Vasculitis There are some bacteria, viruses and fungi that directly affect the endothelium of the blood vessels. The infections involving the blood vessels result in vasculitis.

Fever Due to Cardiac Infections

Infections affecting the cardiovascular system manifest as bloodstream infections.

Pericarditis, myocarditis and endocarditis as well as vegetations on defective valves of the heart lead to bacteremia. This may be transient or continuous. These infections occur when bacteria, fungi or viruses from infectious foci (often the oral cavity) spread through the bloodstream and get attached to damaged areas in the heart. If it is not treated quickly, endocarditis can damage or destroy the heart valves and lead to life-threatening complications.

Endocarditis may develop after dental extraction or IV drug abuse, following foci of infection in the skin or elsewhere.

Individuals with heart valve disorders, congenital heart disease or prosthetic valve replacement are prone to cardiac infections.

Pathogenesis

Colonies of bacteria and cell fragments at the site of the infection called vegetations break loose and travel to other parts of the body such as the brain, lungs, abdominal organs, kidneys or limbs and produce microthrombi. Endocarditis is the most common cardiovascular infection worldwide. It can cause several major complications.

Etiology

- **Pericarditis** It could be viral (ECHO, Adeno) or bacterial (*S. aureus, H. influenzae, S. agalactiae* and *M. tuberculosis*). Rarely, amebic pericarditis is caused by *Entamoeba histolytica*. Chagas disease (African trypanosomiasis) may cause myocarditis or pericarditis.
- **Endocarditis** Infective endocarditis is invariably associated with vegetations on the valves, caused by *Staphylococcus aureus*, HACEK group, nutritionally- deficient streptococci, *Bartonella* species, *Legionella* species, *Tropheryma whipplei*, *Mycobacterium* species and fungi (candida from prosthetic valve vegetations).
- **Myocarditis** Most common causes are of viral origin—Coxsackievirus, Epstein–Barr virus, cytomegalovirus, hepatitis and HIV. Bacterial causes include *Chlamydia, Mycoplasma, Treponema,* streptococci and staphylococci.

Clinical Features of Infective Endocarditis

Breathlessness, fever, chest pain and arrhythmias are the common features. Clinical examination for cardiac signs, ECG and ECHO help in the diagnosis.

Diagnosis of Infective Endocarditis

Diagnosis is done based on some cardinal findings known as the Duke criteria.

Duke criteria

Major criteria

i) Positive blood culture

ii) Evidence of endocardial involvement—ECHO findings—intracardiac mass on valve, abscess or new partial dehiscence

iii) New valvular regurgitation

Minor criteria

i) Fever (>100°F)

ii) Predisposing heart condition or IV drug abuse

iii) Vascular phenomenon, e.g., arterial emboli

iv) Immunologic phenomenon, e.g., Osler's nodes, Roth's spot
v) Microbiological—culture or serologic evidence of infection
vi) Infective endocarditis evidenced by (a) two major, (b) one major and three minor or (c) five minor criteria

Laboratory Diagnosis of Infective Endocarditis

Pericardial fluid (in pericarditis) and blood culture confirms an etiological diagnosis.

Multiple blood cultures need to be done because there may be transient bacteremia.

Paired sample collected thrice (with a gap of one hour in between each collection), the total duration not exceeding 24 hours, is the ideal method for isolating bacteria from blood culture in infective endocarditis.

The other modality of diagnosis is echocardiography (transtracheal or transesophageal echocardiography).

Treatment

Infection is eradicated by eliminating the bacteria from the vegetations. Hence treatment has to be done with bactericidal antibiotics for a prolonged period. High serum concentrations of the antibiotics have to be achieved, hence they are given parenterally so that they reach the vegetations in adequate concentrations.

Specific antibiotics are prescribed based on the organisms isolated by culture and their sensitivity.

INFECTIONS OF THE BLOODSTREAM

When microorganisms invade the bloodstream, the host mounts a systemic response to these organisms. This manifests as:

- Septicemia
- Bacteremia

Septicemia refers to the presence of microbial antigens and endo- or exotoxins in the bloodstream. Sepsis refers to the response the host mounts against these products. It occurs when there is active bacterial multiplication and release of the products (toxins) in the bloodstream,

causing harm to the host.

Bacteremia indicates the presence of a focus of disease or may represent the transient presence of bacteria in the bloodstream without multiplication. Bacteria may be recovered from 20–40% of patients by blood culture.

Bacteremia may be transient, continuous or intermittent:

- Transient bacteremia may occur spontaneously, with minor events such as brushing of teeth or chewing of food or by procedures like manipulation of infected tissues, instrumentation of contaminated mucosal surfaces and surgery involving non-sterile sites.
- Continuous bacteremia is associated with bacterial endocarditis, endovascular infections and septic shock, wherein organisms are released into the bloodstream at a fairly constant rate.
- Intermittent bacteremia, as seen with the causative agents of meningitis, pneumonia, pyogenic arthritis and osteomyelitis, are often recovered from blood early in the course of these diseases. In the case of a sequestered focus of infection, such as an abscess, bacteria are released into the blood approximately 45 minutes before a febrile episode.

Pathogenesis

Host response to microbes in the bloodstream

The microbial agents are recognised by the host's macrophages and neutrophils. The antigens (e.g., lipopolysaccharide or LPS) are bound to toll-like receptors (TLR), which trigger the production of cytokines, chemokines and leukotrienes. These exert endocrine, paracrine and autocrine effects, which in turn result in systemic inflammatory response syndrome (SIRS) with fever or hypothermia, leucocytosis or leucopenia, tachypnea or tachycardia.

Pathophysiology of shock

Septic shock is mediated by activated mononuclear cells producing cytokines, such as tumour necrosis factor (TNF) and interleukins (IL). Gram-negative bacteria release endotoxin, which is a lipopolysaccharide

(LPS). The core of LPS, lipid A, mediates a systemic response which includes fever and the activation of complements and certain clotting factors. Gram-positive bacteria produce exotoxins which generate a similar response.

Clinical Presentation

The two main categories of bloodstream infections are **intravascular** (from within the cardiovascular system, e.g., infective endocarditis, myocarditis and vasculitis) and **extravascular** (those entering the blood circulation through the lymphatic system from the site of infection, e.g., salmonellosis and urosepsis which originates in the urinary tract).

These occur when the infection in the primary site is overwhelming or not controlled or when the immunity of the host is compromised, as is seen in patients who have AIDS or those on immunosuppressive therapy.

Signs and symptoms of bacteremia/septicemia include fever or hypothermia, chills, hyperventilation and subsequent respiratory alkalosis. Septic shock is characterised by fever, acute respiratory distress, shock, renal failure and intravascular coagulation and can be initiated by exotoxins or endotoxins.

Etiology

The etiological agents of bloodstream infection are listed in Table 60.1.

Table 60.1 Common etiological agents of bloodstream infections

Bacteria	Viruses	Fungi	Parasites
Staphylococcus aureus	HIV	*Candida*	*Plasmodium*
Coagulase-negative staphylococci (device-associated)	Epstein–Barr	*Cryptococcus*	*Trypanosoma*
Beta-hemolytic streptococci			
Enterococcus species	Cytomegalovirus	*Coccidioides immitis*	*Babesia*
S. pneumoniae (associated with pneumonia or meningitis)		*Histoplasma capsulatum*	*Wuchereria*
Viridans streptococci (infective endocarditis)		*Mucor* species	
Salmonella species (enteric fever)			

Escherichia coli		*Blastomyces dermatitidis*	*Loa loa*
Klebsiella pneumoniae		*Aspergillus* species	
Enterobacter species			
Proteus species			
Pseudomonas species (mainly HAI)			
Brucella species (brucellosis)			
HACEK group of organisms (infective endocarditis)			
Other organisms isolated from blood —*Leptospira*, *Burkholderia* (melioidosis), *Yersinia* (plague)			

Figure 60.1 is an algorithm of the approach to the diagnosis of bloodstream infections.

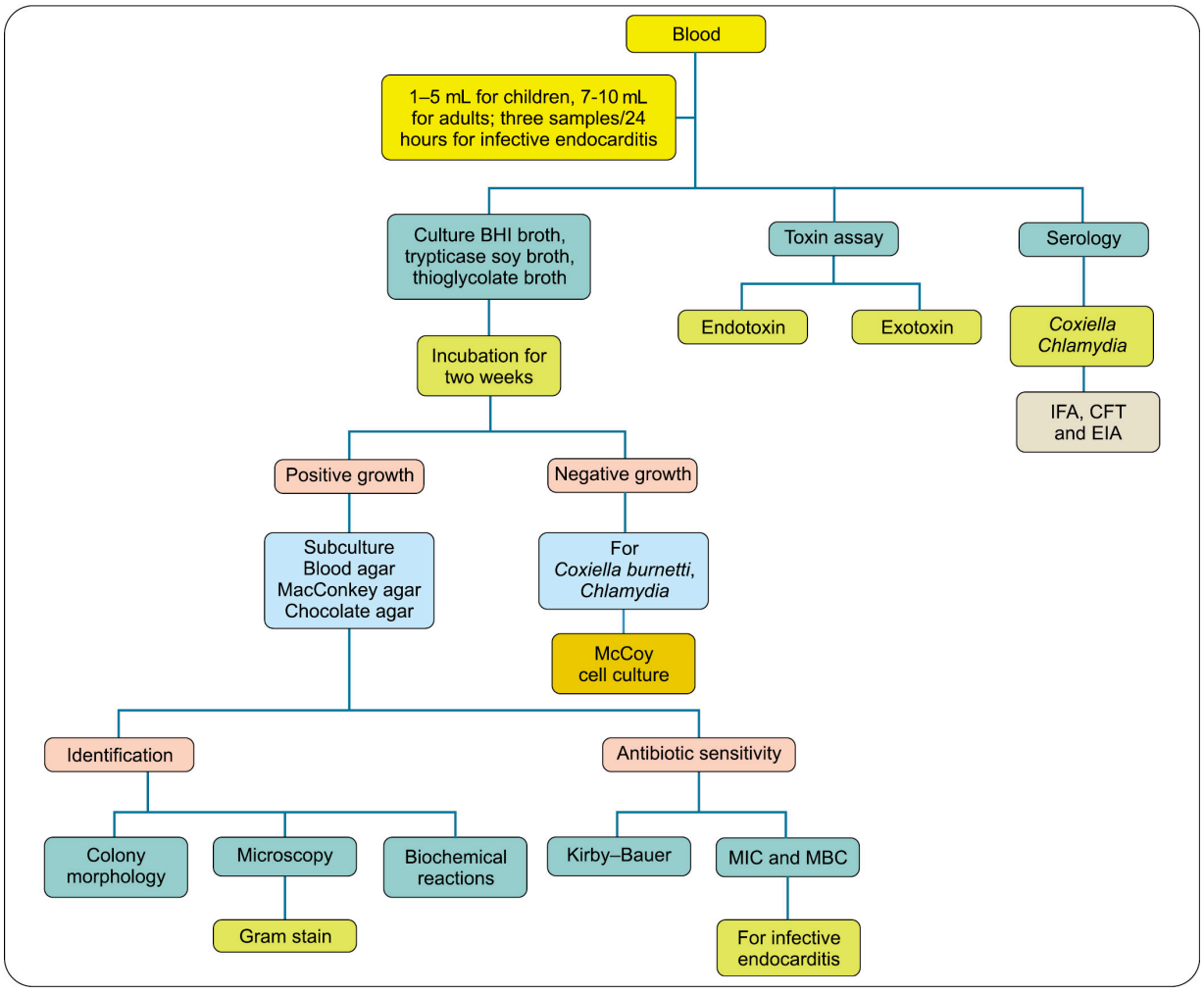


Fig. 60.1 Approach to the diagnosis of bloodstream infections (BHI—Brain heart infusion broth)

Laboratory Diagnosis

Diagnosis depends on the isolation of the causative organism from blood.

1. Preparation The skin at the site of blood collection should be cleaned with 70% isopropyl alcohol before collecting the sample. Strict asepsis must be ensured to prevent skin flora from contaminating the blood sample.

2. Specimen For infants and small children, 1–5 mL of blood is drawn for culture. In adults, 7–10 mL of blood per culture is recommended. In patients with suspected infective endocarditis, three samples within a 24-hour period are collected (due to intermittent bacteremia). Samples should be collected before antimicrobial therapy is initiated.

3. Culture In the conventional blood culture method, blood obtained by

aseptic venipuncture is inoculated in blood culture media, for example, brain-heart infusion (BHI) broth, trypticase soy broth or thioglycolate broth. Incubation is at 37°C for two weeks. Subcultures are made on MacConkey agar, blood agar and chocolate agar after 24 hours, 48 hours and on the 7th day of incubation. Sometimes, it may be necessary to incubate further if fungal or other slow-growing bacteria are suspected. In the automated systems, blood is inoculated in commercial culture bottles and incubated in the machines. Any growth of microorganisms is detected and flagged by the system and the respective bottle is removed. Subcultures are then made by conventional methods.

The HACEK bacteria, certain fungi and *Brucella* are slow-growing and may require incubation for up to two weeks. *Coxiella burnetii* and *Chlamydia* species do not grow in cell-free culture media.

Blood must be collected from a peripheral vein in patients with intravenous lines. To rule out catheter-related bloodstream infection (CRBSI), a sample from the IV line and another from the peripheral vein need to be collected, where both the samples will grow the same organism. Differential time to positivity (culture

from catheter flags earlier, at least 2 hours before the culture from peripheral vein OR by quantitative blood cultures, a colony count of microbes grown from blood obtained through the catheter hub that is at least 3-fold greater than the colony count from blood obtained from a peripheral vein) determines CRBSI.

Fungal infections can be identified by inoculation into Sabouraud dextrose broth or the automated blood culture bottles. These also support the growth of yeasts and yeast-like organisms.

If malaria or leishmaniasis (presenting with other related symptoms) is suspected, the peripheral blood smear or bone marrow aspirate must be collected. Leishmania can be isolated by culture on NNN medium (Novy-MacNeal-Nicolle medium), Schneider's or Trager's medium.

4. Identification The bacterial and yeast organisms isolated by the culture are further identified by colony morphology, Gram staining and biochemical reactions (known as phenotypic characters). Automated rapid identification systems like VITEK, Phoenix and MALDI ToF-MS are being increasingly used by several laboratories to decrease the reporting time.

Parasitic infections can be confirmed by peripheral or bone marrow smear examinations using Leishman's or Giemsa smear.

In case of bacterial infection, **antibiotic susceptibility** test is performed

by the Kirby–Bauer disc diffusion method. In addition, it is necessary to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) as a guide to treatment (adequate dose of antibiotic) in infective endocarditis and other life-threatening infections. MIC-based dose adjustment for personalised antibiotic therapy is an emerging concept. Bacterial infections are treated based on the MIC, which exhibits variations in the response to antibiotics tested by the disc diffusion methods. MIC can be obtained directly by E-test or the VITEK automated system or kit-based microbroth dilution tests.

5. Serology It is a convenient and commonly-used method for the identification of *Coxiella burnetii* and *Chlamydia*. The tests available are indirect immunofluorescent antibody (IFA), complement fixation test (CFT) and enzyme immunoassay (EIA).

6. Other tests

- Total count—leucocytosis
- Erythrocyte sedimentation rate (ESR)—elevated
- Procalcitonin—raised
- C-reactive protein—detectable
- Urine—culture may be positive
- Chest X-ray—lung infiltrates in pneumonia
- Echocardiography for the detection of vegetation

Detection of bacterial endotoxin—Limulus lysate test—is a sensitive test for the detection of bacterial endotoxin.

FEVER OF UNKNOWN ORIGIN

Fever is defined as an elevation of body temperature by pyrogens (substances that stimulate cytokines to induce fever). These substances induce host leucocytes and macrophages to release fever-producing mediators such as the cytokine, i.e., interleukin-1. These raise the set point of the hypothalamic thermoregulatory centre through the action of prostaglandin E2 so that there is an increase in body temperature.

The purpose of fever is not completely understood but small elevations in body temperature appear to enhance immune function and inhibit pathogen

growth.

Fever can be a manifestation of several underlying causes. It could be due to infectious or non-infectious causes.

Fever of unknown origin or FUO (as classified by Durack and Street)

"**Classic FUO** The patient exhibits temperature > 38.3°C (101°F) on several occasions, has had the illness for more than three weeks and a diagnosis has not been established despite a week of in-patient investigation.

Nosocomial fever In addition to temperature > 38.3°C, the patient has been hospitalised for ≥ 24 hours but had no fever or was not incubating a fever on admission, and has undergone evaluation for at least three days.

Neutropenic fever In addition to temperature > 38.3°C and evaluation for at least three days, the patient has a neutrophil count ≤ 500 per mm^3.

HIV-associated fever In addition to temperature > 38.3°C and evaluation for at least three days, the patient has had the fever for > 4 weeks (out-patient) and > 3 days (in-patient) and HIV infection has been confirmed."

Etiology

Some of the common causes of fever that have infectious etiology are presented in Table 60.2. Bacterial break down products including proteins (exotoxins)and lipopolysaccharide toxins released from bacterial cell membranes act on the hypothalamus either directly or through interleukin-1, causing fever. Fever due to non-infectious disorders such as myocardial infarction, pulmonary emboli and neoplasms are caused by the injured or abnormal cells that incite the production of pyrogens. Some of the causes are listed in Table 60.3.

Table 60.2 Infectious causes of fever of unknown origin (FUO)

Bacterial	Viral	Fungal	Parasitic
M. tuberculosis	Cytomegalovirus	*Candida albicans*	*Plasmodium* spp.
Salmonella spp.	Epstein–Barr virus	*Cryptococcus neoformans*	*Leishmania* spp.
Brucella spp.	Arboviruses	*Histoplasma capsulatum*	*Trypanosoma* spp.
Chlamydia psittac	Enteroviruses	*Aspergillus* spp.	*Toxoplasma gondii*

Leptospira spp.	HIV	*Coccidiodes immitis*	*Wuchereria bancrofti*
Rickettsia spp.			*Brugia malayi*
Coxiella burnetti			
Mycoplasma spp.			
Atypical mycobacteria			
Burkholderia pseudomallei			

Table 60.3 Non-infectious causes of FUO

Neoplasms	Connective tissue disorder	Granulomatous diseases
Lymphoma Leukemia Myeloma Renal cancer Colon cancer Liver cancer Metabolic disorder Gout Porphyria	Systemic lupus erythematosus (SLE) Polyarteritis nodosa	Crohn's disease Sarcoidosis Rheumatoid arthritis Polymyositis

Clinical Presentation

Fever may present as follows:

- As an **intermittent fever**, where the patient's temperature returns to normal at least once every 24 hours. This type of fever is associated with gram-negative or gram-positive sepsis, abscesses and infective endocarditis.
- As **remittent fever,** where the patient's temperature does not return to normal, although it varies a few degrees in either direction. Remittent fevers are associated with viral upper respiratory tract, legionella and mycoplasma infections.
- As **sustained** or **continuous fever,** where the patient's temperature remains above normal with minimal variations (usually less than 1°F or 0.55°C). These fevers may be caused by drugs.

- As **recurrent or relapsing fever,** which consists of one or more episodes of fever, each as long as several days, with one or more days of normal temperature between episodes. Relapsing fevers can be caused by various infectious diseases, including Borrelia and chronic fungal infections.
- As **undulant fever** as seen in brucellosis, where the temperature rises and falls over a period of time.

Approach to Diagnosis of FUO

Figure 60.2 is an algorithm of the steps in the diagnosis of FUO due to infectious and non-infectious causes.

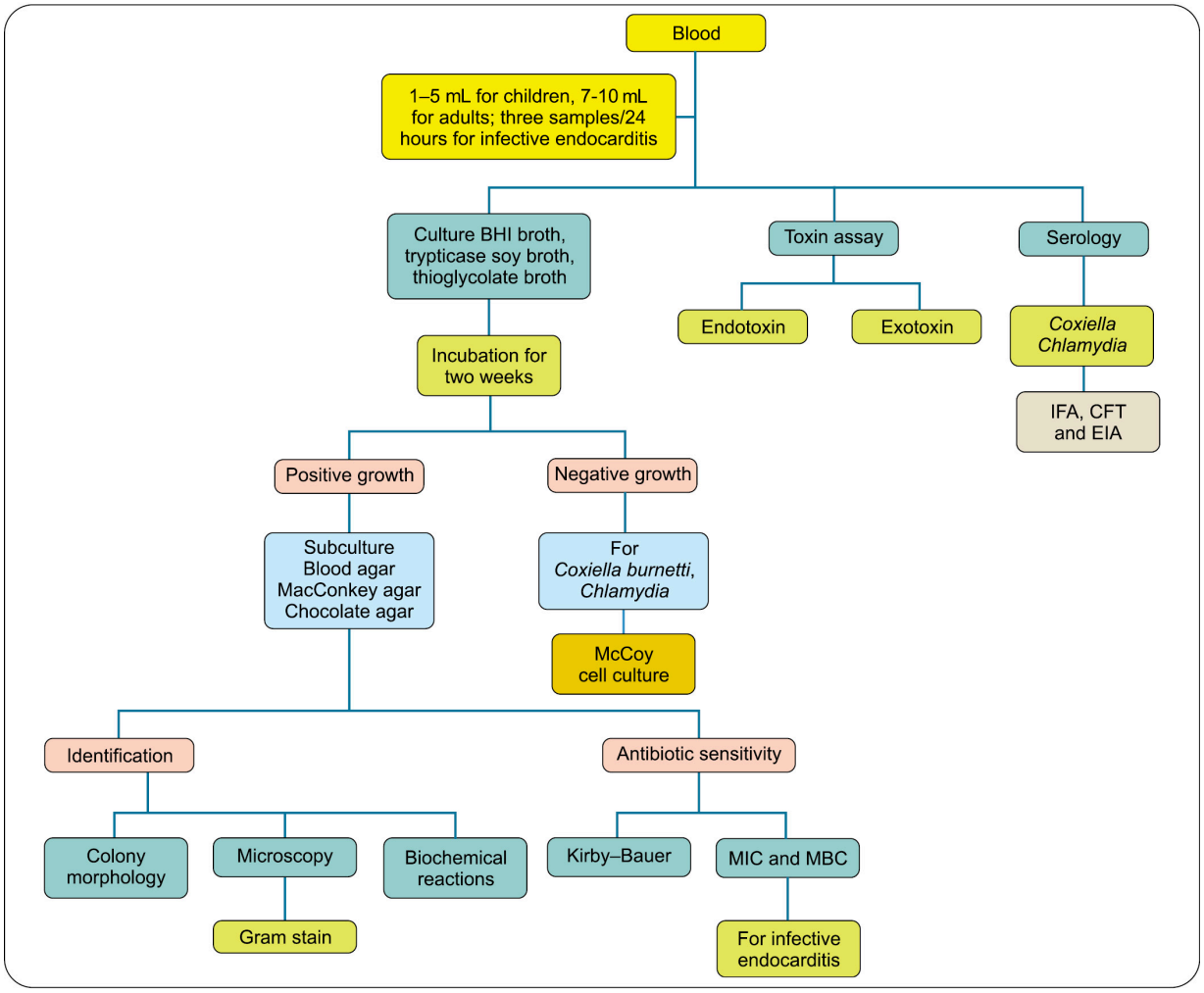


Fig. 60.2 Algorithm for diagnosis of FUO (BA—blood agar, MA—MacConkey agar, CA—chocolate agar, ASO—antistreptolysin O, NTM—non-tuberculous mycobacteria, SLE—systemic lupus erythematosus)

Collection of Specimens

Prior to specimen collection, a complete clinical history (including details of travel, immunisation and exposure to diseases) should be obtained, followed by a detailed physical examination; information from the history and examination may influence the choice of specimen taken for processing.

Blood For complete blood counts (CBCs), erythrocyte sedimentation rate (ESR), microscopy (wet films and Leishman-stained smears), culture and serological tests

Urine For urinalysis and culture

Bone marrow For biopsy and culture

Material from abnormal tissues, temporal artery, lung and abdominal viscera are used for biopsy.

Diagnostic Procedures

Tests that may suggest or confirm that an infection is the cause of FUO are as follows:

- CBCs and erythrocyte sedimentation rate—for example, neutrophilic leucocytosis in CBC may suggest a pyogenic infection; markedly elevated ESR may suggest a tuberculous etiology.
- Microscopy of blood—for example, a wet film may demonstrate microfilariae or trypanosomes; a stained smear may demonstrate malarial parasites, trypanosomes and microfilariae.
- Culture of blood—by conventional or automated blood culture systems, e.g., for enteric (salmonella) or other pathogens (organisms causing infective endocarditis).
- Serological tests
    - ELISA for the diagnosis of **viral diseases** [e.g., HIV, cytomegalovirus (CMV) or Epstein–Barr virus (EBV) infections]
    - CFT for certain **bacterial diseases** [lymphogranuloma venereum (*C. trachomatis* serotypes L1-L3), psittacosis (*C.*

psittaci), tularemia (*Francisella tularensis*), Q fever (*Coxiella burnetii*)]
    - ASO titre for rheumatic fever
    - ELISA/SAT for brucellosis (*Brucella* species)
    - ELISA/Weil–Felix for rickettsia
    - Paul–Bunnell for infectious mononucleosis
    - Widal test for typhoid fever
    - IF/RA and other autoimmune markers for rheumatoid arthritis
- Microscopy of appropriate specimen
    - Wet mount for *Entamoeba* (liver abscess)
    - Gram stain for gram-negative bacilli, *Brucella* species
    - Ziehl–Neelsen stain for *Mycobacterium* species
    - Giemsa stain for fungus
- Culture
    - Urine for pyogenic bacteria or tubercle bacilli
    - Bone marrow for fungus and Leishmania
- Antibiotic sensitivity test is performed for all bacterial isolates.
- Chest X-ray may demonstrate the presence of pulmonary tuberculosis.
- Echocardiography may demonstrate findings indicative of rheumatic fever or infective endocarditis.
- Skin tests—delayed hypersensitivity reactions may suggest that the FUO is due to tuberculosis (Mantoux skin test) or a fungal infection (e.g., the histoplasmin skin test for histoplasmosis).
- Tests that may suggest or confirm other (non-infective) causes for the FUO.
- Serological tests that detect, for example, antinuclear antibody or anti-DNA antibody (suggestive of SLE), anti-thyroglobulin antibody (suggestive of subacute thyroiditis) or tests to detect other collagen vascular diseases.
- Imaging studies such as chest X-rays, echocardiograms, upper and lower gastrointestinal tract barium studies, ultrasound, CT and MRI scans, intravenous urograms, and scans of lung, bone

and other tissues to detect neoplastic processes or collagen vascular diseases.
- Biopsies from bone marrow (to detect leukemia), lymphoid tissue (Hodgkin's disease), gastrointestinal tract structures by endoscopy, temporal artery, lung and other tissues.
- Molecular tests—multiplex PCR may be performed to detect bacterial or viral nucleic acids. If tuberculosis is suspected, nucleic acid amplification assays should be performed.

Fever Due to Infections Transmitted by Blood Transfusion

Blood transfusion is a possible source of infection. Several agents can potentially be transmitted through blood transfusions, including bacteria, viruses and parasites. Although not strictly through transfusion, intravenous injections of drugs and other substance abuse results in bloodstream infections, the most common being hepatitis B, C and HIV.

Transfusion-transmitted bloodstream infection can be classified based on the causative organisms as follows:

- Viruses—hepatitis B, hepatitis D, hepatitis C, HIV, cytomegalovirus and human T-cell lymphotropic virus
- Bacteria—*Treponema, Pseudomonas, Staphylococcus, Yersinia enterocolitica, Serratia* and *Flavobacterium*; incidence is common in blood components rather than whole blood preparations
- Parasites—Plasmodium

Potential sources of infection include intrinsic contamination due to donor bloodstream infection, contamination of blood containers or needles and contamination of processing equipment.

Incidence of infections transmitted by blood transfusion have come down considerably due to stringent quality control, screening of blood for potential pathogens and regulatory licensing of blood banks.

GASTROINTESTINAL AND HEPATOBILIARY TRACT

INFECTIONS

The gastrointestinal tract is colonised by normal flora which, along with mucosal immunity, defend the host. Infections are caused by the ingestion of exogenous pathogens in sufficient quantities to evade the immune mechanism of the host. Ingested pathogens produce disease by production of toxins, invasion and multiplication. Infection of the GIT can lead to diarrhea, dysentery or food poisoning.

Acute diarrheal diseases are the leading cause of morbidity and mortality in children in the developing countries, next only to respiratory infections.

DIARRHEA

Diarrhea is an increase in fluid frequency or volume of bowel movement relative to the usual habit of the individual (generally more than 3–5 episodes of liquid/loose stool per day). It may be of the following types:

- Acute diarrhea with or without vomiting is the predominant symptom of infective gastroenteritis. Acute diarrhea may be watery or bloody (due to inflammation).
- Chronic diarrhea is generally non-infectious (due to inflammatory bowel disease and pancreatic enzyme deficiency).
- Traveller's diarrhea refers to the passing of watery stool 3 or more times in 24 hours, often seen in travellers.

Gastroenteritis refers to inflammation of the stomach and intestines, resulting from bacterial invasion or toxins or viral infection. It manifests with vomiting and diarrhea.

The pathogenic mechanisms of gastroenteritis are presented in Table 60.4.

Dysentery (acute bloody diarrhea) is the presence of blood and mucus in stool, often with tenesmus.

Diarrhea in children Diarrheal disease is the second leading cause of death in children under five years of age. It results from contaminated food and water sources. The most common etiological agents are Rotavirus and *E. coli*. Diarrhea in children leads to severe dehydration and death. Long-term diarrhea leads to malnutrition.

Table 60.4 Pathogenic mechanism of gastroenteritis

	Toxins		
	Enterotoxins	**Cytotoxins**	**Neurotoxins**
Pathogenesis	Cause outpouring of electrolytes and fluid into the lumen of intestine, resulting in profuse watery diarrhea	Disrupt the intestinal epithelium, leaving raw, unprotected mucosa The resulting inflammatory response causes neutrophils and blood to be shed in stool	Result of ingesting toxins produced by microorganisms
Disease	Diarrhea	Dysentery	Food poisoning or intoxication
Organisms	*V. cholerae* Non-cholera vibrios Enterotoxigenic *E. coli* *Salmonella* species *Campylobacter jejuni* *Clostridium perfringens* *Clostridium difficile* *Bacillus cereus* *Giardia lamblia*	*Shigella dysenteriae* Enterohemorrhagic *E. coli* *Clostridium difficile* *Staphylococcus aureus* *Clostridium perfringens* *Entamoeba histolytica*	*Staphylococcus aureus* *Bacillus cereus* *Clostridium perfringens* *Clostridium botulinum*

Clinical Presentation

There are three clinical types of diarrhea:

- Acute watery diarrhea—lasts several hours or days, and includes cholera
- Acute bloody diarrhea—generally occurs in the early phase of dysentery
- Persistent diarrhea—lasts 14 days or longer

Small intestinal pathology—manifests as large volume, watery stools (enteritis), without pus or blood (unaided eye and microscopy).

Large intestinal pathology—usually manifests as frequent, small-volume stools with pus and/or blood (unaided eye and microscopy).

Etiology

Etiological agents responsible for infective diarrheas are listed in Table 60.5.

Table 60.5 Etiology of infective gastroenteritis

Diarrhea	**Traveller's diarrhea**	**Dysentery**	**Food poisoning**
Vibrio cholerae	*E. coli* (ETEC)	*Shigella* species	*Staphylococcus aureus*

E. coli (EIEC, EPEC, EHEC)	*Salmonella* Norwalk virus, Rotavirus *Giardia lamblia*	*Campylobacter* species	*Bacillus cereus*
Salmonella species		*E. histolytica* *G. lamblia* *B. coli*	*C. botulinum*
C. difficile		*F. hepatica* *T. saginata* *T. solium* *H. nana* *T. trichiura*	
Yersinia enterocolitica			
Rotavirus Norwalk virus Adenovirus Calicivirus Coronavirus Astrovirus Enterovirus			
Candida albicans (antibiotic-associated diarrhea)			
Cryptosporidium *Isospora belli*			

Approach to Diagnosis of Infective Diarrheas

Figure 60.3 is an algorithm of the steps in the diagnosis of infective diarrheas.

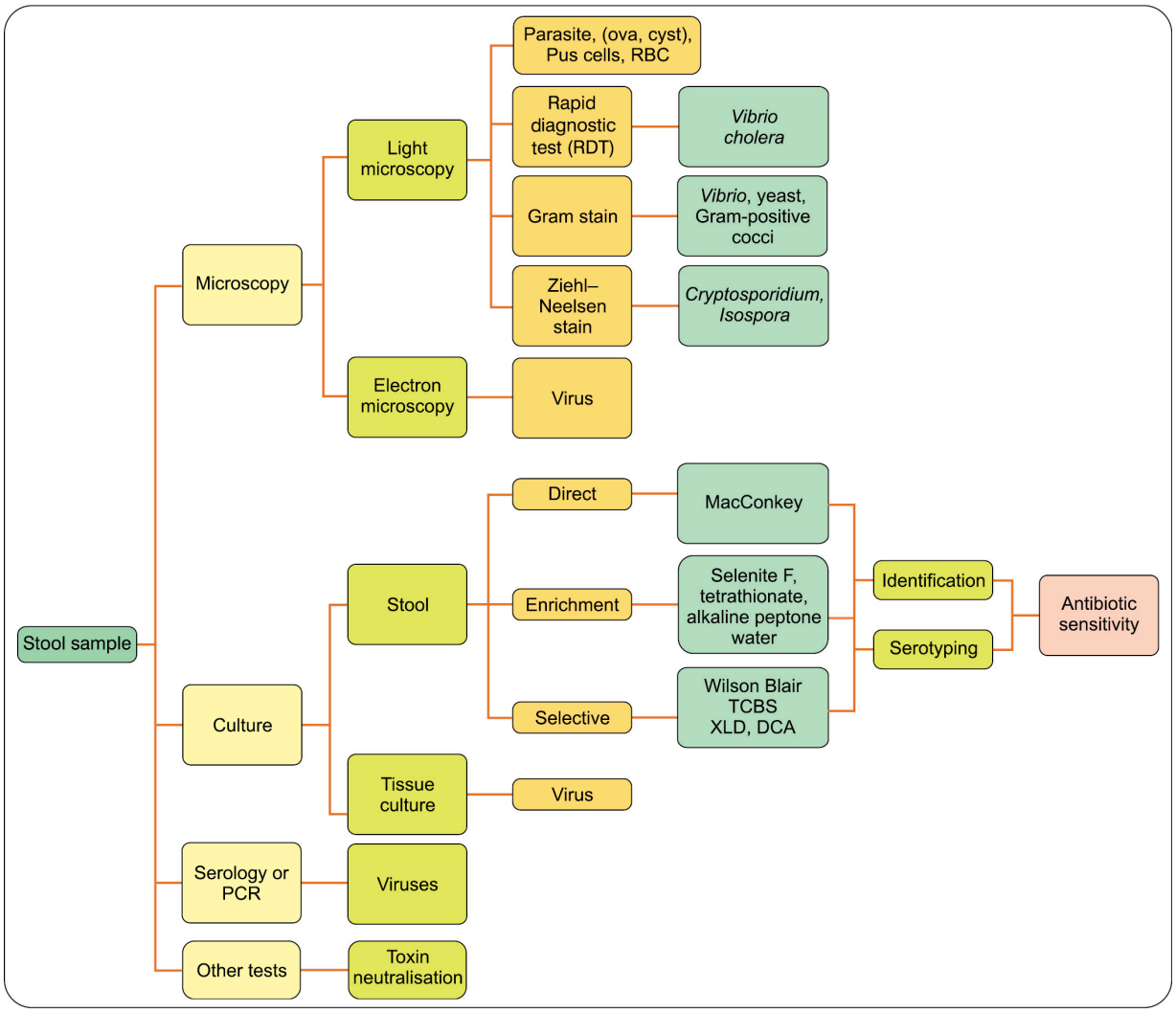


Fig. 60.3 Algorithm for the diagnosis of infective diarrheas (TCBS—thiosulfate-citrate-bile salts-sucrose agar, XLD—xylose lysine deoxycholate and DCA—deoxycholate citrate agar)

Laboratory Diagnosis

1. Specimen collection A sterile screw-capped, wide-mouthed container is used to collect feces for culture. If the collection of feces is not possible, a rectal swab can be submitted. For an outbreak of food poisoning, the food implicated and the vomitus of the patient are collected along with the feces sample.

2. Transport Fresh feces are ideal, but a transport medium such as Cary–Blair or Venkatraman–Ramakrishnan is used if a delay in transport to the laboratory is anticipated. For *Vibrio cholerae*, alkaline peptone water, which also serves as an enrichment medium, can be used for transport.

3. Microscopy

- **Wet preparation** Microscopy of feces is done to detect pus cells and RBCs, and ova or cysts of parasites.
- **Saline and iodine preparation** Microscopy of a wet preparation of feces is also done to detect helminth ova, protozoan cysts and protozoan trophozoites.
- **Gram-stained smear** This is helpful only in certain situations because feces contains many bacteria in a healthy person. Such situations include:
    - Presence of curved bacilli suggestive of *Vibrio*
    - Presence of yeasts in an immunocompromised host or an infant
- **ZN stain** Modified acid-fast stain can be used for the identification of *Cryptosporidium*, *Isospora* and *Cyclospora*.
- **Electron microscopy** is done for the identification of viruses.

4. Culture of feces

- **Direct culture** Here, the media used are non-selective, e.g., MacConkey agar, or selective, e.g., xylose-lysine-deoxycholate (XLD) agar, deoxycholate citrate agar (DCA) or thiosulphate citrate bile salt sucrose agar (TCBS).
- **Enrichment culture** Fresh feces are introduced into a liquid culture medium. For example, selenite F broth, tetrathionate broth (incubated for 12–18 hours) or alkaline peptone water (6–8 hours). Following this, a subculture is made on the solid culture medium used for direct plating. In this method, if the sample contains only a small number of pathogens, the enrichment medium allows selective growth of the pathogen, which then becomes easier to isolate.

5. Identification The organism is identified by biochemical tests and serotyping.

Antibiotic sensitivity test is carried out for *Shigella*, *Vibrio* and *Salmonella* isolates.

Tissue culture is used for the culture of viruses. ETEC penetrate HeLa and HEp-2 cells in culture. EHEC verotoxin (VT) can be detected by cytotoxic effect on Vero cells.

6. Serology ELISA is used for the detection of *E. coli* O157 : H7 (EHEC), Shiga toxin and *C. difficile* toxins. In addition, it can be used to detect rotavirus antigens. PCR using specific primers can be used to detect the Norwalk virus.

Detection of enterotoxin Immunodiffusion, ELISA, neutralisation and latex agglutination tests can be used for the detection of toxins.

Rapid diagnostic test (RDT) *V. cholerae* O1 and O139 antigens are detected in stool specimen using monoclonal antibodies, by the immunochromatographic method.

A dipstick is dipped into a tube containing the stool sample and applied on the well of the immunochromatographic cassette. A colour band denotes the presence of the antigen (O1 or O139).

Treatment

The mainstay of the treatment of acute diarrhea is fluid and electrolyte replacement to maintain hydration. Viral diarrheas are self-limiting. Infective bacterial diarrheas may require appropriate antibiotics like ceftriaxone, ciprofloxacin and tetracycline. When parasites are the cause, metronidazole and tinidazole may be used to treat individuals.

A word of caution while treating diarrhea with antibiotics—antibiotics may increase the risk of hemolytic uremic syndrome in children with *E. coli* O157, H7(Shiga toxin-producing *E. coli*). Prolonged use of antibiotics may also lead to the development of resistance in the normal gut flora in addition to the pathogen.

FOOD POISONING

Food poisoning is an acute manifestation of diarrhea (diarrheic type) or vomiting (emetic type) caused by toxins produced by microorganisms. It is suspected in an outbreak when it follows the consumption of food from a common source, and the examination reveals a non-inflammatory etiology of diarrhea.

Toxin-mediated food poisonings have a short incubation period since the

toxins are pre-formed in the food, e.g., staphylococcal and *Bacillus cereus* food poisoning have an incubation period of 1–6 hours. Diarrhea, abdominal cramps, nausea and vomiting are common symptoms.

Food poisoning due to *Clostridium perfringens* has an incubation period of 8–14 hours. It is caused by heat-resistant clostridial spores in tinned or processed food. Diarrhea, vomiting and abdominal cramps are the common features.

Non-infectious etiology of food poisoning can be due to toxins of some fish and shellfish.

HEPATOBILIARY INFECTIONS

Many systemic infectious pathogens including hepatotropic and non-hepatotropic microorganisms can infect the liver through both the systemic and portal circulation. Infections can be due to bacteria, viruses and parasites.

Some examples of primary hepatotoxic microbes are:

- Bacteria—*Leptospira*
- Virus—hepatitis virus (hepatitis A, B, C, D, and E viruses); yellow fever virus, EBV and CMV are also important causes of hepatitis.
- Parasites—*Plasmodium*, *Entamoeba*, *Schistosoma, Fasciola hepatica* and *Clonorchis sinensis*.
- Infections of the gallbladder (cholecystitis) can be caused by enteric pathogens including *E. coli*, *Klebsiella* and *Enterobacter* species. *Proteus* and *Enterococcus* are the other common agents causing cholecystitis.

Clinical Presentations of Hepatitis

The main clinical features of hepatitis are loss of appetite, mild-to-severe pain in the right upper quadrant and vomiting in the early phase of the infection. This is followed by jaundice with yellow discolouration of the conjunctiva, skin (may not be appreciable in dark-skinned persons) and urine. Arthralgia, fatigue and malaise may be other complaints. Invariably, low-grade fever is also present. In leptospirosis, the patient may have rashes

in addition to fever.

Laboratory Diagnosis

Liver enzymes are almost always altered. Serum amylotransferases and aspartate amylotransferases are elevated. There is an increase in bilirubin levels coinciding with the appearance of jaundice.

Specific etiological diagnosis depends on the cause of hepatitis.

Diagnosis depends mainly on serological evidence of infection. Viral hepatitis is diagnosed by the detection of viral antigens and antibodies by ELISA, immunochromatographic methods, and estimation of viral load by RT-PCR.

Bacterial etiology such as *Leptospira* infections can be detected by serology or culture of blood on EMJH medium.

Management

Symptomatic management is commonly done for uncomplicated infections. Anti-viral treatment is given to prevent chronicity and complications. Pegylated IFN and tenofovir and entecavir are some of the recommended drugs for hepatitis C and B.

Hepatitis due to bacterial causes is treated with appropriate antibiotics (e.g., penicillin or cephalosporins for leptospirosis).

(These have been discussed in detail in the chapters that deal with the individual organisms.)

SKIN, SOFT TISSUE AND MUSCULOSKELETAL INFECTIONS

Skin, soft tissue and musculoskeletal infections may be caused by organisms invading the body from the external environment through breaks in skin, by burns, injury, bites or surgery (primary), or from organisms that reach the site through the blood as a part of a systemic disease (secondary).

INFECTIONS OF THE SKIN (DERMIS AND EPIDERMIS)

Types

- Impetigo (involves the epidermis and occurs often on the face, mainly in children)
- Erysipelas (involves the dermis as an erythematous painful lesion)
- Cellulitis (affects the deep dermis; it is a diffuse erythematous lesion without a clear margin)
- Folliculitis (involves the hair follicle; it is a localised papule containing pus)
- Abscess (furuncle, carbuncle or boil) (affects the deep dermis and is present as an inflamed nodule with a central region that is purulent with a raised point)

Skin infections can also be grouped as follows:

- Vesicles—viral infections, zoster, shingles, hand, foot and mouth disease
- Bullae—necrotising fasciitis, gas gangrene
- Papular and nodular lesions—dracunculiasis, creeping eruptions, larva migrans
- Crusted lesions—impetigo, ringworm

Subcutaneous tissue infections may manifest as:

- Necrotising fasciitis—soft tissue infections involving the fascia and muscles overlying soft tissues caused by group A streptococci, *S. aureus*, *Bacteroides* and *Clostridium* species.
  Progressive bacterial synergistic gangrene, which is usually a post-operative complication following a thoracic or abdominal surgery, caused by *S. aureus*, *Proteus* and anaerobic streptococci.
- Myositis—may vary from necrosis of muscle to necrotising cutaneous myositis or anaerobic myonecrosis.

Etiology

Etiological agents of infections of the skin, subcutaneous tissue and various types of wounds are listed in Tables 60.6, 60.7, 60.8 and 60.9.

Table 60.6 Etiology of infection of the epidermis and dermis

Bacteria	Virus	Fungi	Parasites
Streptococcus pyogenes (Erysipelas) (Impetigo) (Cellulitis)	Herpes zoster Varicella-zoster Herpes simplex	Dermatophytes *Sporothrix schenckii*	Cutaneous larva migrans (*A. braziliensis*)
Staphylococcus aureus (Impetigo) (Cellulitis) (Furuncle)			
Corynebacterium minutissimum (Erythrasma)			

Table 60.7 Etiology of infections of subcutaneous tissue

Bacteria	Fungi	Parasites
Clostridium perfringens *Clostridium novyi* *Clostridium septicum* *Bacillus* species *Bacteroides* species *Peptostreptococcus* species *Staphylococcus aureus* Group A streptococci Some of the above bacteria cause necrotising fasciitis and myositis	*M. mycetomatis* *M. grisea* *Candida* species	*Trichinella* *Taenia solium* *W. bancrofti* *D. medinensis*

Table 60.8 Etiology of post-operative wound infections

Bacteria	Fungi
• *S. aureus* • **Coagulase-negative staphylococci** • *Streptococcus pyogenes* • *Proteus* **species** • *Pseudomonas* **species** • *Bacteroides* **species** • *Prevotella* **species**	*Candida* **species**

Table 60.9 Etiology of burn wounds

Bacteria	*Fungi*
S. epidermidis Enterobacteriaceae *Pseudomonas* species *Proteus* species *Bacteroides* species	*Candida* species *Aspergillus* species

Approach to Diagnosis of Skin and Soft Tissue Infections

Figure 60.4 is an algorithm of the steps in the approach to the diagnosis of skin and soft tissue infections.

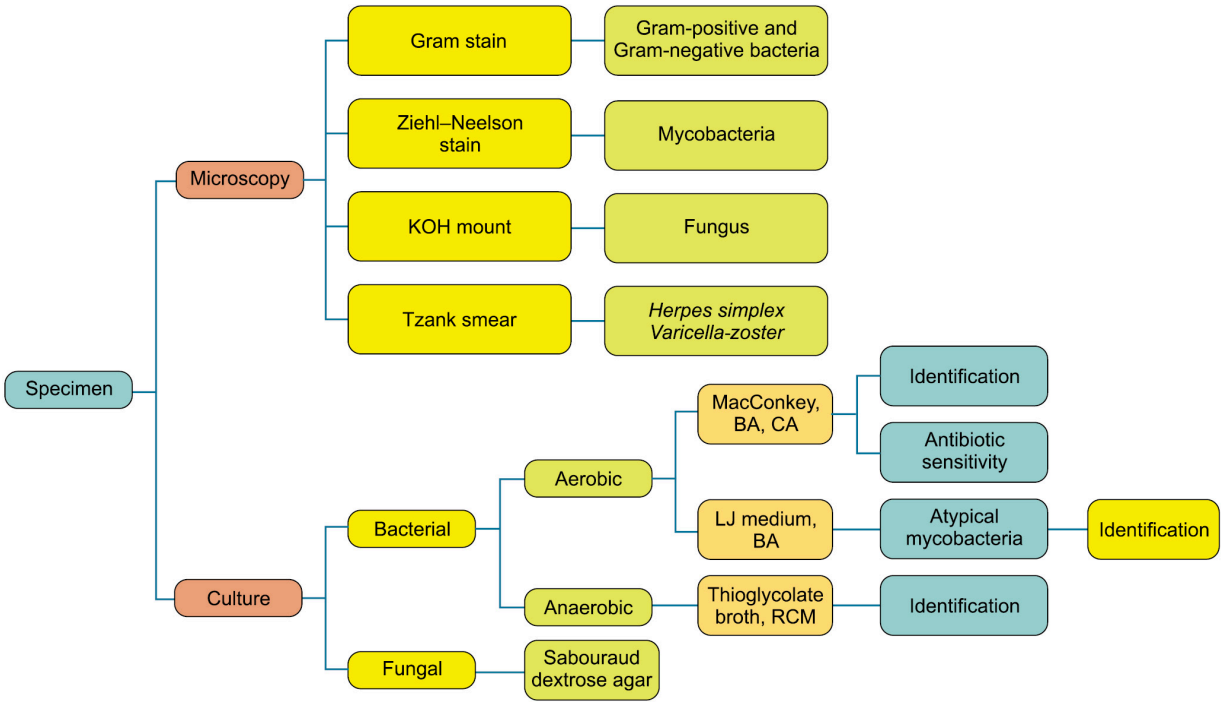


Fig. 60.4 Approach to the diagnosis of skin and soft tissue infections (BA—Blood agar, CA—cetrimide agar, RCM—Robertson's cooked meat)

Laboratory Diagnosis

1. Specimen

- Pus from the wound is collected by:
  - Incision and drainage
  - Needle aspiration
  - Sterile swab

- Vesicle fluid is collected by:
  - Needle aspiration
- Dermatophyte infection:
  - Scrapings from the active border of the lesion
- Erysipeloid:
  - Skin biopsy
- Subcutaneous infection:
  - Sample is collected from the base of the lesion
  - Surgical biopsy of deep tissues is done to obtain a positive culture

2. Microscopy Gram stain is routinely performed on all samples. KOH mount is done for suspected dermatophyte infection. Tzanck smear is carried out on vesicle fluid for the detection of herpes simplex and varicella-zoster infections.

3. Culture For aerobic bacteria, specimens are inoculated onto blood agar, MacConkey and chocolate agar. For atypical mycobacteria, LJ medium is inoculated. For anaerobic culture, thioglycolate broth and Robertson's cooked meat medium are inoculated and incubated in a Gaspak or anaerobic jar. Sabouraud dextrose agar is used with and without cycloheximide to isolate fungal pathogens.

4. Identification This is done on the basis of colony morphology, Gram/ZN stain and biochemical reactions.

Antibiotic sensitivity test is performed by the Kirby–Bauer disc diffusion method for bacterial and yeast isolates.

Treatment

Most superficial skin infections are treated by draining the localised area of pus collection. Local dressing with topical antibiotics or oral cephalexin or dicloxacillin often suffice for small superficial infections.

Cellulitis, erysipelas, deep tissue infections, necrotising fasciitis (which may be a medical emergency requiring surgical debridement), myositis, etc., require systemic antibiotics (intravenous), e.g., clindamycin, piperacillin tazobactam and vancomycin (for MRSA).

BONE AND JOINT INFECTIONS

These are serious infections as they may lead to the destruction of the bone and cartilage, leading to disability.

Two major categories of infection are:

i) Osteomyelitis

ii) Infectious arthritis (septic arthritis)

Osteomyelitis

Acute osteomyelitis is generally of bacterial etiology and **chronic** is of either fungal or tubercular etiology. Acute osteomyelitis in children often involves the ends of long bones.

Clinical manifestations in acute osteomyelitis are abrupt-onset bone pain and localised tenderness at the site of infection. Chronic infection is of insidious onset. The bone is necrosed and sequestrum (avascular necrotic bone) is formed.

The most common route of entry of the organisms to the bones is by hematogenous spread.

They may also enter by trauma through penetrating injury. The most common cause of acute osteomyelitis is *Staphylococcus* and that of chronic osteomyelitis is *M. tuberculosis* (Pott's disease). In prosthetic-associated osteomyelitis, the causative organism is *S. epidermidis.*

Salmonella species may cause osteomyelitis in sickle cell anemia.

Septic Arthritis

Bacteria may invade the joint space and cause infectious arthritis. Large, weight-bearing joints like the knee or hip are often involved. The source of infection is often from the skin, reaching the site through the bloodstream.

Acute onset with fever, joint pain, redness and swelling are the cardinal features. *Staphylococcus aureus* is the most common cause of septic arthritis. The cause of septic arthritis may be *N. gonorrhoeae* in sexually active individuals (knee, wrist, ankle, or elbow may be involved). Other causative organisms include *S. epidermidis* and other coagulase-negative *s*taphylococci in prosthetic joints.

Laboratory Diagnosis

Bone sequestrum (in osteomyelitis) and joint aspirates (in arthritis) are stained to demonstrate bacteria and polymorphs. They are cultured on blood agar to yield the organism. Blood culture may yield the organism in around 30% of acute cases of osteomyelitis and septic arthritis.

Treatment

Empiric treatment with vancomycin and cefazolin must be started early to prevent damage to the bone. Intravenous antibiotic treatment may be necessary in acute osteomyelitis and septic arthritis. Removal of joint fluid by arthrocentesis may be needed in addition to antibiotic therapy.

CENTRAL NERVOUS SYSTEM INFECTIONS

Central nervous system (CNS) infections are common medical emergencies. Prompt diagnosis and appropriate management are the key to controlling high rates of morbidity and mortality associated with CNS infections. These infections affect all age groups. However, they are of special concern in neonates, immunocompromised hosts and the elderly.

The infections can be classified as:

- Meningitis, which may be acute or chronic
- Encephalitis
- Focal infection in the brain—brain abscess and subdural empyema

Microorganisms associated with infections of the CNS are bacteria, viruses, parasites, fungi and prions.

The major route of entry of these organisms is through the bloodstream from a focus elsewhere in the body. The microorganisms enter by crossing the blood–brain or blood–CSF barrier. However, a contiguous spread from the nasopharynx or suppurative lesions of the middle ear to the meninges can also occur through the olfactory and cranial trigeminal nerves. Other nerves may also be involved. Penetrating trauma to the skull may also lead to infections in the brain.

MENINGITIS

Meningitis is an inflammation of the meninges, the membrane covering the brain and spinal cord. When a microorganism (usually a bacterium or virus) enters the subarachnoid space, there is an inflammatory response in the meninges. Bacterial meningitis is usually preceded by nasopharyngeal or middle ear colonisation, followed by (i) microbial invasion of the tissue and intravascular space and (ii) bacteremia.

They may be classified as acute or chronic depending on the speed of the initial presentation and subsequent progress. Most bacteria cause acute pyogenic meningitis, while *Mycobacteria* and *Cryptococcus* produce chronic meningitis.

Clinical Presentation

Symptoms Headache, fever and altered sensorium are the usual symptoms.

Signs The classical triad of fever, headache and neck rigidity characterise acute pyogenic meningitis. Positive signs for meningism are Kernig's sign (hip and knee are flexed at 90° and then the knee is extended, which produces pain) and Brudzinski's sign (flexion of the neck causes flexion of the knees). Altered mental symptoms or seizures in children are other signs of meningitis. Infants and young children may have a high or low body temperature, be irritable or drowsy or have a poor appetite.

Clinical case presentations of meningitis due to *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Bacteroides fragilis* (brain abscess), *Haemophilus influenzae*, *Mycobacterium tuberculosis*, Herpes simplex virus (aseptic meningitis) and poliovirus 1 (aseptic meningitis) have been described in earlier chapters.

Etiology

The etiological agents of meningitis are listed in Table 60.10.

Etiologically, meningitis may be:

- Pyogenic meningitis of bacterial origin (caused by pyogenic organisms), which may be categorised into four types based on the age groups affected as that occurring in neonates, in children, in adults and in the elderly
- Tuberculous (caused by *M. tuberculosis*)

- Aseptic meningitis caused by viruses and categorised according to viral and non-microbial causes

Table 60.10 Causes of meningitis

Bacteria	Viruses (aseptic meningitis)	Fungi	Parasites
Neonates and infants • *E. coli* • Group B Streptococci *(Streptococcus agalactiae)* • *Staphylococcus aureus* • *H. influenzae* • *Streptococcus pneumoniae* • *Klebsiella* species • *Listeria monocytogenes*	• Enteroviruses (ECHO, coxsackie, polio) • Paramyxoviruses (mumps, measles) • Herpesviruses (herpes simplex, varicella-zoster) • Adenoviruses • Arboviruses (flavivirus, bunyavirus)	• *Cryptococcus neoformans* • *Candida albicans* • *Aspergillus* species • *Histoplasma capsulatum* • *Coccidioides immitis*	• *Entamoeba histolytica* • *Naegleria* • *Acanthamoeba* • *Toxoplasma gondii*
Children • *Haemophilus influenzae* • *Neisseria meningitidis* • *Streptococcus pneumoniae*			
Adults • *Neisseria meningitidis* • *Streptococcus pneumoniae*			
Elderly • *Streptococcus pneumoniae* • *Staphylococcus aureus* • Gram-negative enteric bacilli			

Bacteria	Viruses (aseptic meningitis)	Fungi	Parasites
Neonates and infants • *E. coli* • Group B Streptococci *(Streptococcus agalactiae)* • *Staphylococcus aureus* • *H. influenzae* • *Streptococcus pneumoniae* • *Klebsiella* species • *Listeria monocytogenes*	• Enteroviruses (ECHO, coxsackie, polio) • Paramyxoviruses (mumps, measles) • Herpesviruses (herpes simplex, varicella-zoster) • Adenoviruses • Arboviruses (flavivirus, bunyavirus)	• *Cryptococcus neoformans* • *Candida albicans* • *Aspergillus* species • *Histoplasma capsulatum* • *Coccidioides immitis*	• *Entamoeba histolytica* • *Naegleria* • *Acanthamoeba* • *Toxoplasma gondii*
Children • *Haemophilus influenzae* • *Neisseria meningitidis* • *Streptococcus pneumoniae*			

Adults • *Neisseria meningitidis* • *Streptococcus pneumoniae*			
Elderly • *Streptococcus pneumoniae* • *Staphylococcus aureus* • Gram-negative enteric bacilli			

Approach to Diagnosis of Meningitis

Figure 60.5 is an algorithm illustrating the approach to the diagnosis of meningitis.

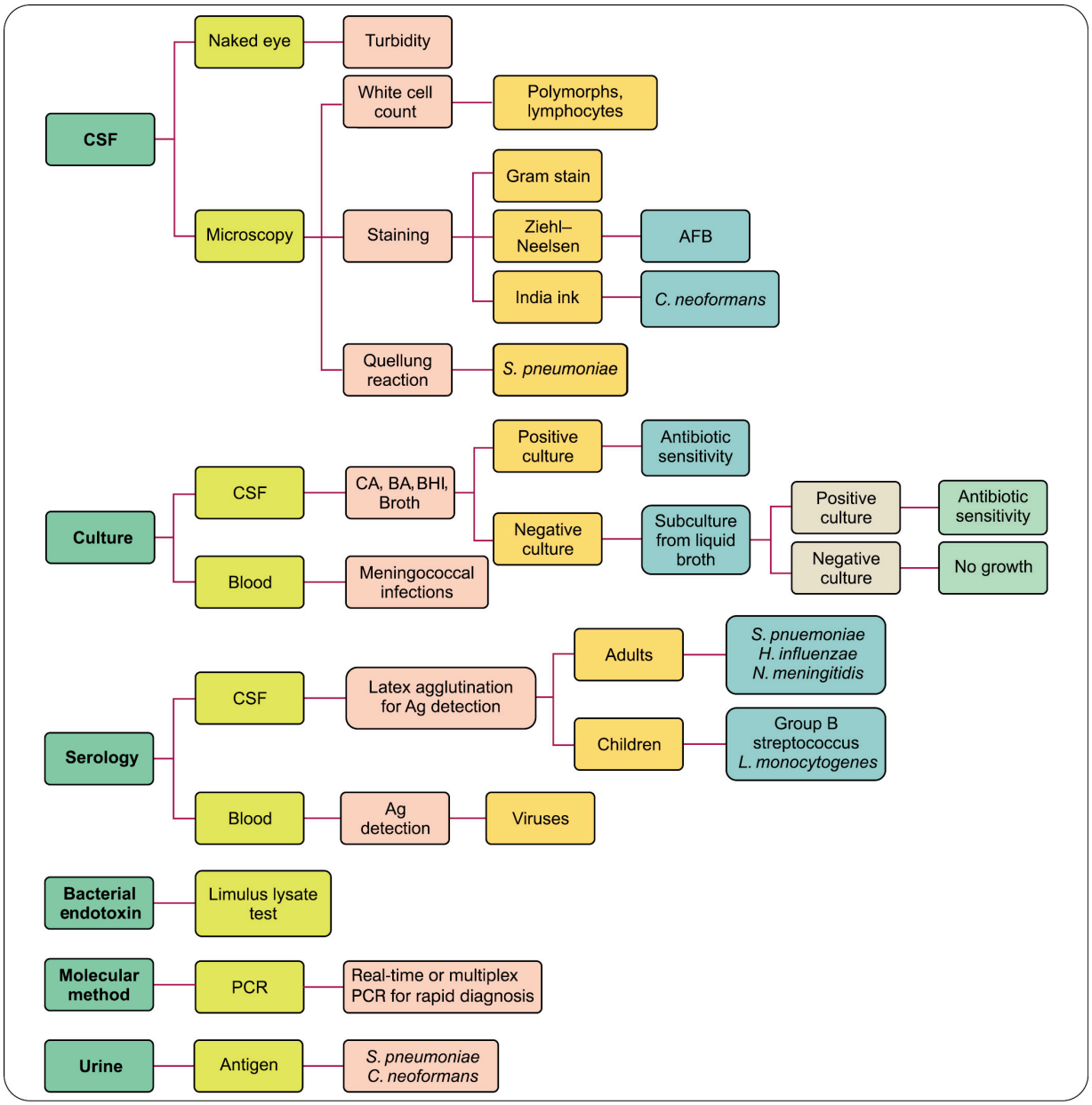


Fig. 60.5 Algorithm of the approach to the diagnosis of meningitis (AFB—acid-fast bacilli)

Collection and Transport of Specimens

CSF is the most appropriate sample. It is obtained by lumbar puncture at the L3-L4 interspace.

A note of caution: Lumbar puncture is NOT performed if there is rise in intracranial pressure.
In addition, it may be necessary to obtain:

- Blood culture (especially in neonates)
- Serum (for viral serology)
- Urine (for antigen detection)

Collection of CSF Under aseptic conditions, CSF is collected in three sterile containers, one each for cell count, biochemical examination and culture.

Transport of CSF The CSF must not be refrigerated when bacteriological examination is required; however, for viral isolation, it must be transported on ice. For bacterial culture, it should be transported at room temperature. In case of delay, the CSF sample should be kept in an incubator at 37ºC and not in a refrigerator.

Laboratory Diagnosis

CSF Examination

Routine (non-microbiological) tests Certain procedures can be performed on the CSF along with microbiological procedures. These may aid in arriving at an etiological diagnosis. These include the appearance of the CSF, total WBC count and lymphocyte count, and estimation of protein and glucose concentration (Table 60.11).

Table 60.11 Interpretation of abnormal results

	White cell count		Biochemistry	
	Neutrophils	**Lymphocytes**	**Protein**	**Glucose**
	(× 106/L)	(× 106/L)	(g/L)	(CSF:blood ratio)
Normal	0	≤ 5	< 0.4	≥ 0.6 (or ≥ 2.5 mmol/L) (>1 month of age)
Normal neonate	0*	< 20	< 1.0	≥ 0.6 (or ≥ 2.5 mmol/L)
Bacterial meningitis	100–10,000 (but may be normal)	Usually < 100	> 1.0 (but may be normal)	< 0.4 (but may be normal)
Viral meningitis	Usually < 100 (but may be normal)	10–1,000 (but may be normal)	0.4–1	Usually normal
TB meningitis	Usually < 100 (but may be normal)	50–1,000 (but may be normal)	1–5 (but may be normal)	< 0.3

* Some studies have found up to 5% of white cells comprise neutrophils in neonates

without meningitis.

Microscopic examination

- Microscopic examination of the sediment of a centrifuged sample of CSF by **Gram stain** is done to detect the presence of microorganisms. A **heaped drop** (one over the other) of the sediment is placed on a dry, grease-free slide without spreading. This enhances the chance of finding small numbers of microorganisms. **Acridine orange** may also be used. Brightly fluorescing microorganisms can be visualised.

  If organisms are not detected, an attempt can be made to demonstrate bacterial antigens by latex agglutination test on the CSF sample or in blood.
- Microscopic examination of a **Ziehl–Neelsen**-stained smear is performed to demonstrate tubercle bacilli; if negative, polymerase chain reaction for detection of *M. tuberculosis* DNA can be done.
- Microscopic examination of an **India ink** preparation of the CSF is done to demonstrate capsulated budding yeast cells of *Cryptococcus neoformans*.

Bacterial culture Chocolate and blood agar are used. The specimen is also inoculated into an enriched liquid medium such as BHI broth since the specimen is likely to contain only small numbers of bacteria which may not grow on solid culture media. After overnight incubation of the liquid culture medium, subcultures can be made from the liquid broth in solid culture media. If there is growth of bacteria in culture, an antibiotic sensitivity test is performed.

Blood culture

This is important when a meningococcal infection is suspected.

Fungal culture is done on Sabouraud dextrose agar or brain heart infusion agar. Two sets of media are inoculated and incubated at 25°C and 37°C, and growth is identified by its morphology. Birdseed agar is used for the culture of *Cryptococcus* species.

Viral culture is done using tissue culture, egg inoculation or animal

inoculation. However, these methods are time-consuming and cumbersome and have been replaced by molecular methods like conventional and real-time PCR.

Serology

Latex agglutination tests for antigen detection This can be done for *S. pneumoniae*, *H. influenzae* and *N. meningitidis*. In neonates, the test for antigens of group B streptococcus (*S. agalactiae*) and *Listeria monocytogenes* may also be performed.

Serological tests for antibodies to different viruses This is done for viruses depending on the clinical diagnosis.

Tests to detect microbial antigen in urine Tests are available to detect *S. pneumoniae* antigen (immunochromatography-based) and *Cryptococcus neoformans* antigen (latex agglutination-based) in urine.

Detection of bacterial endotoxin The **Limulus lysate test** is extremely sensitive for the detection of bacterial endotoxin. The principle of the test is based on the coagulation of horseshoe crab (*Limulus polyphemus*) amoebocytes (blood cells) with blood containing endotoxin.

Molecular methods

To detect microbial nucleic acid For suspected infections due to *M. tuberculosis* and viral infections, PCR-based tests are increasingly being used.

To detect microbial nucleic acid in blood PCR-based tests can also be done on blood samples if CSF-based tests do not yield results.

Treatment

Empiric treatment and supportive management are started immediately.

Following provisional diagnosis based on clinical presentation, an empiric antibiotic with a broad spectrum is started. If bacterial etiology is suspected, ceftriaxone in combination with gentamicin is started till specific results are obtained. Viral meningitis and encephalitis are managed with supportive therapy.

Malaria (cerebral) or encephalitis due to free-living amoebae are managed with chloroquine or natamycin and metronidazole respectively. Fungal meningitis due to *Cryptococcus* is treated with liposomal amphotericin B. Doses of all medications are age- and body weight-dependent and are

generally given by intravenous route for quick delivery.

ENCEPHALITIS

Encephalitis is an acute inflammation of the brain. The major causes of the disease are viruses, a few parasites and bacteria. There may also be immunological causes where the immune system mistakenly causes inflammation of the brain tissue.

The major viral etiological agents are rabies virus, herpesvirus, measles, Epstein–Barr virus, arbovirus group JE, West Nile, WEE, EEE, etc. Parasites that cause this disease are *Acanthamoeba* and *Naegleria*. A type of encephalitis that has bacterial etiology is anthrax meningoencephalitis. Early symptoms are fever, headache and photophobia. It may lead to seizures.

Laboratory Diagnosis

A lumbar puncture for cerebrospinal fluid might reveal higher-than-normal levels of protein and white blood cells. Viral agents may be detected by specific amplification methods by conventional PCR or RT-PCR.

Treatment

Viral encephalitis has limited treatment options. Early laboratory confirmation helps in the treatment of herpes encephalitis by acyclovir.

RESPIRATORY TRACT INFECTIONS

The respiratory tract is the most common site for infection by pathogenic microorganisms because it is in direct contact with the environment and is continuously exposed to microorganisms suspended in the air. Respiratory tract infections may occur in the nasopharynx, oropharynx, middle ear and paranasal sinuses, epiglottis, larynx, trachea, bronchi, bronchioles and lungs.

UPPER RESPIRATORY TRACT INFECTIONS

Upper respiratory tract infections (URTIs) are limited to the nose, pharynx, sinuses and larynx. Infections include:

- Common cold
- Sinusitis
- Croup—inflammation of the larynx, trachea and large bronchi (laryngotracheobronchitis)
- Epiglottitis (most frequently caused by *H. influenzae* type B)
- Pharyngitis (sore throat)—this is the most common presentation, and usually manifests as high-grade fever; other features include cervical lymphadenopathy and the presence of exudates on the tonsillar area
- Tonsillitis, commonly caused by Group A streptococci
- Diphtheria—this is one of the more serious URTIs which may be life-threatening; a pseudo-membrane may be present; bull neck is a feature of diphtheria

Etiology

Etiological agents of respiratory tract infections are listed in Table 60.12.

Table 60.12 Etiological agents of respiratory infections

Bacteria	Viruses	Fungi	
Agents associated with upper respiratory tract infections (URTI)			
Streptococcus pyogenes	Adenovirus	*C. albicans*	
Corynebacterium diphtheriae	Rhinovirus		
Mycoplasma pneumoniae	Influenza virus		
Staphylococcus aureus	Respiratory syncytial virus	*H. capsulatum*	
Agents associated with lower respiratory tract infections (LRTI)			**Parasites**
Haemophilus influenzae	Influenza virus	*B. dermatitidis*	*P. westermani*
Streptococcus pneumoniae	Respiratory syncytial virus	*C. immitis*	*C. sinensis*

Group B streptococci (in neonates and infants)	Parainfluenza virus	*P. jirovecii*	*A. lumbricoides* (larva)
Mycobacterium tuberculosis	Epstein–Barr virus	*Aspergillus* species	*A. duodenale* (larva)
Atypical mycobacteria	Echovirus	*C. neoformans*	*Strongyloides stercoralis* (larva in the immunocompromised)
Pseudomonas aeruginosa	Measles virus	*Aspergillus* species	
Bordetella pertussis	Varicella-zoster virus		
Klebsiella species			

Upper respiratory tract infections are mostly viral and are diagnosed clinically. Laboratory confirmation is required for diphtheria, streptococcal pharyngitis and tonsillitis. The diagnostic algorithm for URTI is given in Fig. 60.6.

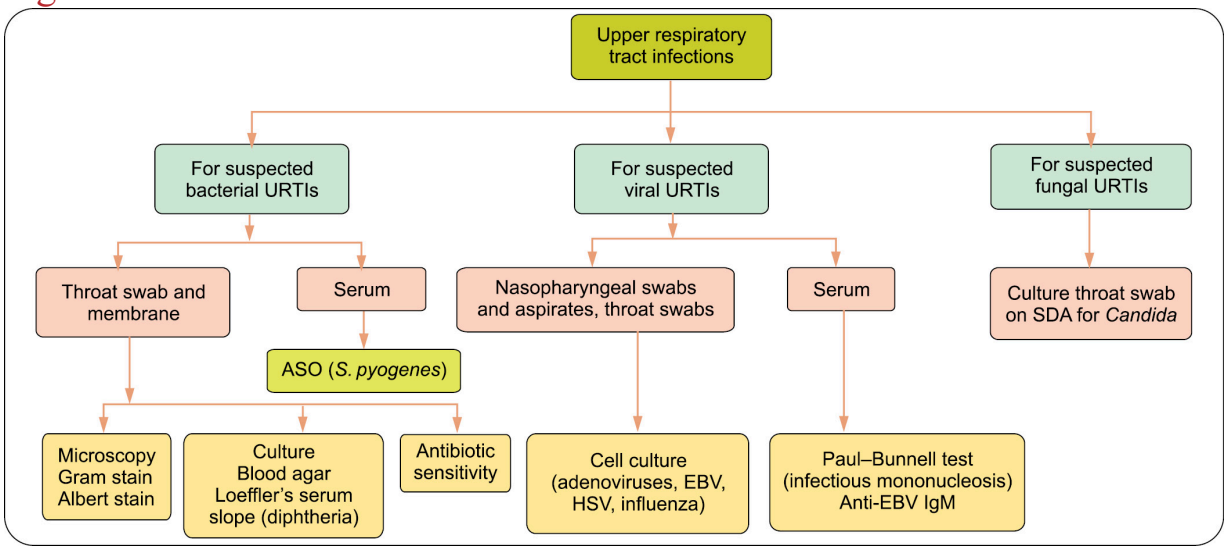

Upper respiratory tract infections
For suspected bacterial URTIs
For suspected viral URTIs
For suspected fungal URTIs
Throat swab and membrane
Serum
Nasopharyngeal swabs and aspirates, throat swabs
Serum
Culture throat swab on SDA for Candida
ASO (S. pyogenes)
Microscopy Gram stain Albert stain
Culture Blood agar Loeffler's serum slope (diphtheria)
Antibiotic sensitivity
Cell culture (adenoviruses, EBV, HSV, influenza)
Paul–Bunnell test (infectious mononucleosis) Anti-EBV IgM

Fig. 60.6 Diagnostic algorithm for upper respiratory tract infections (ASO—antistreptolysin O, EBV—Epstein-Barr virus, HSV—herpes simplex virus, SDA—Sabouraud dextrose agar)

Collection and Transport of Specimens

For infections of bacterial etiology Two throat swabs should be collected: one for direct microscopic examination and another for culture from the tonsillar pillars. If diphtheria is suspected, a part of the membrane must be taken for processing.

For infections of viral etiology Nasopharyngeal swabs, aspirates or throat washings are collected. Calcium alginate or Teflon-coated swabs are used.
Serum is collected when streptococcal pharyngitis or infectious mononucleosis is suspected.

Laboratory Diagnosis

Albert stain This is used to detect metachromatic granules in *C. diphtheriae* infections.

Gram stain This is not very useful in URTI, which is mostly of viral etiology, and does not differentiate the pathogen from normal flora. However, in diphtheria, the smear may show abundant gram-positive rods in cuneiform arrangement.

Immunofluorescence may help in the rapid identification of diphtheria.

However, most of the above direct demonstrations from clinical specimens are unreliable due to the presence of normal microbial flora of the throat.

Culture Inoculation is done on BA (5% sheep BA) and incubated under 5% CO_2 overnight. This will allow the recovery of most bacterial pathogens. For diphtheria, culture on Loeffler's serum and potassium tellurite agar is carried out. SDA medium is used for *Candida albicans*.

Treatment

Specific antibiotic for bacterial URTI

- For streptococcal sore throat, penicillin G, amoxicillin and erythromycin are used (dose is dependent on age and body weight).
- If viral URTI is suspected, antibiotics should be avoided. This is one instance where antibiotics are most misused, giving rise to antibiotic resistance.

LOWER RESPIRATORY TRACT INFECTIONS

Lower respiratory tract includes the bronchi, bronchioles, alveoli, lobes of the lungs, pleura and pleural cavity. The clinical entities of infections of the

lower respiratory tract include:

- **Bronchitis** It is a self-limiting infection of the bronchi, commonly viral in etiology. It is of acute onset with dry cough which may later progress to an expiratory wheeze. The common causes are influenza A and B, coronavirus, respiratory syncytial virus
  and human metapneumovirus. Infection subsides in 1–2 weeks.
- **Bronchiolitis** This involves the bronchioles. It is seen in children < 2 years. The infection is caused by RSV, influenza, corona and metapneumovirus. Bacteria are generally not involved. The epithelial cells of the bronchioles are damaged by the virus. Bronchiolitis is also self-limiting.
- Pneumonia involves the alveoli. The syndromes may present as acute (community-acquired or nosocomial), sub-acute or chronic (pulmonary tuberculosis, fungal, aspiration pneumonia and lung abscess) diseases and as pneumonia in immunocompromised individuals. Diagnosis is clinical.
- Other localised infections in the lower respiratory tract and lungs include lung abscess and bronchiectasis.
- Lung abscess is a localised infection of the lung parenchyma, which may result in a cavity. On radiological examination, an air-fluid interface is noted. The common feature of lung abscesses is the aspiration of the oropharyngeal bacteria. Anaerobic bacteria (*Peptostreptococcus, Fusobacterium*) are a predominant cause of lung abscesses.
- Seeding from the bloodstream is often due to pyogenic organisms, mainly *Staphylococcus aureus* from a right-sided endocarditis.
- Other chronic airway diseases are:
  - **Bronchiectasis** Pockets in the airways where mucus gets trapped are likely to get infected. This may follow tuberculosis, pneumonia or chronic obstructive pulmonary disease (COPD).
  - **Empyema** This is an infection of the pleural cavity with the presence of exudative effusion in the cavity (pus in the

pleural cavity).

Clinical Presentation

In **adult acute community-acquired pneumonia**, the main presenting features are cough, fever and chest pain. Causative organisms are usually *S. pneumoniae,*
H. influenzae or *Klebsiella*. **Acute health care-associated (nosocomial) pneumonia** tends to occur in comatose patients, those with left ventricular failure or who have been intubated or anesthetised, and those who are on the ventilator (**ventilator-associated pneumonia – VAP**). In **chronic pneumonia**, the main presenting features are chronic cough, fever, night sweats and weight loss. In **lung abscess**, the main presenting features are chest pain, cough and fever.

Collection and Transport of Specimens

Sputum is a good sample and is easy to collect. If cough is present but not productive, the patient should be asked to first inhale warm hypertonic saline vapour to induce the production of sputum, which is then collected.

If the patient is not able to provide a sputum sample, an endotracheal aspirate or bronchoalveolar lavage (BAL) sample must be collected from the lower respiratory tract. If the patient is on a ventilator or intubated, a non-bronchoscopic lavage or BAL sample may be collected to diagnose VAP.

Figure 60.7 is an algorithm of the approach to the diagnosis of pneumonia.

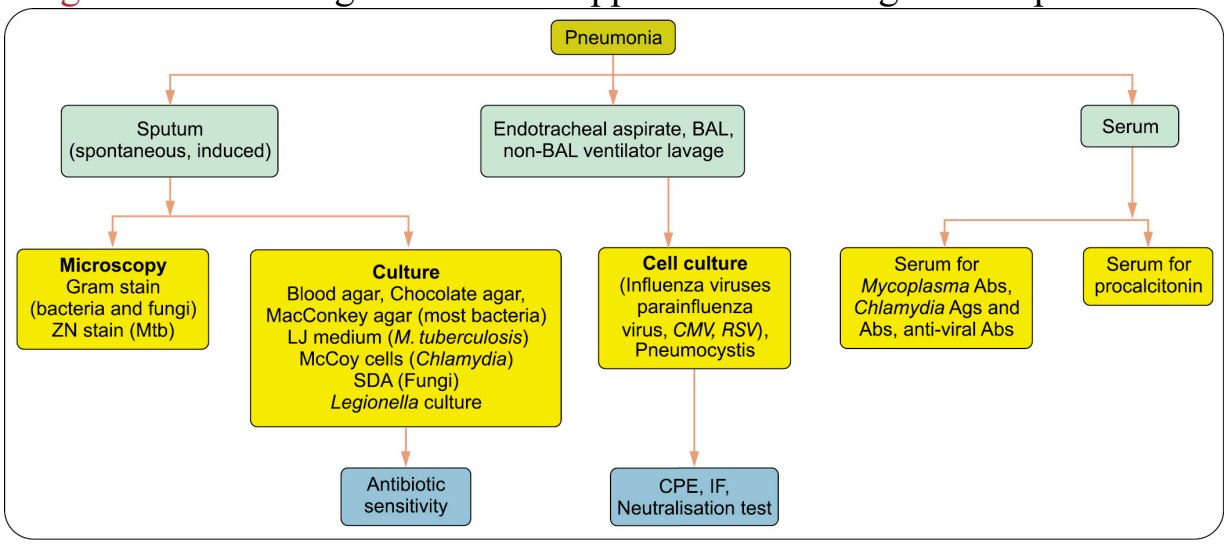


Fig. 60.7 Algorithm of the approach to the diagnosis of pneumonia (BAL—bronchoalveolar lavage, CPE—cytopathic effect, IF—immunofluorescence)

Laboratory Diagnosis

1. **Specimen** Sputum, broncho-alveolar lavage, endotracheal aspirate or pleural or rarely, lung aspirate in infants and neonates with pneumonia are the specimens. Blood is the ideal specimen to isolate the organism by culture.

2. Microscopy

Gram stain An acceptable specimen is that with <10 squamous epithelial cells per low-power field (100×) and the presence of ≥25 polymorphonuclear leucocytes per 100× field.

- *Ziehl–Neelsen stain* Acid-fast bacilli (AFB) are suggestive of tuberculosis.
- Giemsa stain This is used for the detection of *P. jirovecii*.

3. Culture This is done on BA (5% sheep BA) and incubated under 5% CO_2 overnight. For diphtheria, culture on Loeffler's serum and potassium tellurite agar is carried out. SDA medium is used for *Candida albicans*.

Antibiotic sensitivity test This is done if *S. pneumoniae* is isolated to detect penicillin-resistant *S. pneumoniae*; if *S. aureus* is isolated in health care-associated pneumonia (HAP), the test is done to detect to detect methicillin-resistant *S. aureus* (MRSA); if *K. pneumoniae* is isolated in HAP, the test is done to detect extended-spectrum beta-lactamases (ESBLs) which confer resistance to many antibiotics. The antibiotic sensitivity test is also done for other organisms isolated in VAP and HAP.

4. Serology To make a serological diagnosis of infection by *Mycoplasma pneumoniae*, specific tests using mycoplasmal antigens can be used as they are very sensitive. If these are not available, the streptococcus MG agglutination test (significant titre ≥ 1:20) or cold agglutination test (significant titre ≥ 1:32) can be performed.

To make a diagnosis of infection due to chlamydiae, tests that detect antigen or antibody can be used.

Chlamydial antigens, particularly lipopolysaccharide antigen, can be

detected by microimmunofluorescence (micro-IF) or ELISA tests. Molecular methods are also being increasingly used.

Procalcitonin is another biomarker which is elevated in invasive pneumococcal disease, including pneumonia, and the levels are monitored to determine prognosis and response to treatment.

Radiological findings often help to differentiate bacterial including tubercular, fungal, viral and atypical pneumonia. With characteristic lung infiltrates, consolidation, cavity and/or patchy pneumonitis, a radiologist can often arrive at a presumptive diagnosis. This is often correlated with other laboratory and clinical parameters to confirm the etiology of pneumonia.

GENITOURINARY AND SEXUALLY TRANSMITTED INFECTIONS

This group of infections, which affect the genital and urinary systems together, are a major cause of morbidity in the adult population worldwide. These infections are age- and gender-specific, as well as based on the socio-behavioural characteristics of the population.

The two major categories of infections in this group are:

i) Urinary tract infections
ii) Sexually transmitted infections

URINARY TRACT INFECTIONS

The urinary tract, starting from the calyces of the kidneys to the urethra, is lined with a sheet of epithelium that is continuous with that of the skin. Although the flow of urine and the sloughing of these epithelial cells serve to protect the urinary tract from infection, microorganisms, particularly bacteria, may enter the urinary tract through the potential pathway of the epithelial surface to cause infection. Urinary tract infections (UTIs) are an important cause of illness, affecting all age groups.

While all portions of the urinary tract may be affected, the most common UTIs are those of the bladder (cystitis) and the renal pelvis or the kidneys (pyelonephritis).

Types of UTI

Infections of the urinary tract can be anatomically classified as:

- **Upper UTI**, involving the kidney or ureter and includes the following conditions:
    - Acute pyelitis—infection of the pelvis of the kidney
    - Acute pyelonephritis—infection of the parenchyma of the kidney
- **Lower UTI**, involving infection from the urinary bladder downwards and includes the following conditions:
    - Urethritis—infection of the urethra
    - Acute urethral syndrome (in women, it is often caused by *Chlamydia*)
    - Cystitis—infection of the urinary bladder
    - Prostatitis—infection of the prostate

Predisposing Factors

- **Age** Incidence increases with age.
- **Gender** Infant boys not circumcised are at a higher risk of UTI than girls. After infancy, girls are at higher risk than boys.
- **Sex** Young females and sexually active females are specifically prone to the infection due to the short urethra, proximity to the anus and urethral trauma during intercourse.
- **Pregnancy** Dilatation of the ureters and renal pelvis, stasis, incompetence of the vesico-urethral valves and hormonal changes increase the risk of UTIs during pregnancy.
- **Structural and functional abnormality of the urinary tract**
    - Obstruction due to urethral stricture, calculus, prostatic hypertrophy and tumour
    - Neurogenic bladder
    - Vesico-urethral reflux
    - Genital prolapse
- **Metabolic** Diabetes mellitus increases the risk of UTIs.
- **Intervention** Instrumentation including catheterisation and any surgical procedure may result in a UTI.

Pathogenesis

Bacteria establish infection by ascending from the anterior urethra through the column of urine. They generally get eliminated by voiding. However, if there is stasis of the urine either in the bladder or ureters due to neurogenic or mechanical cause, the microorganisms colonise and establish infection. Hematogenous spread to the urinary tract occurs in less than 2% of cases. The factors that interplay are host factors (gender, age and familial predisposition), microorganism [presence of type 1 pili (fimbriae)] or adhesins, e.g., uropathogenic *E. coli* and environmental factors (vaginal colonisers, pH, anatomic and functional abnormalities, prostatic hypertrophy, presence of stones, etc., and catheterisation or any instrumentation).

Clinical Presentation

Asymptomatic bacteriuria About 5–7% of pregnant women have been reported to have urinary infection without any symptoms. Such asymptomatic bacteriuria, if undetected and untreated, may lead to symptomatic infection, pyelonephritis and hypertension in the woman, as well as prematurity and perinatal death of the fetus.

Symptomatic UTI The common symptoms include urgency and frequency of micturition, associated with discomfort or pain. Pyelonephritis is characterised by loin pain, tenderness, high fever and rigour.

Common presentation of cystitis or lower UTI is dysuria, fever with chills and frequency. In upper UTI (pyelitis or pyelonephritis), patients present with fever and flank pain. In infants and children with UTI, fever, vomiting, diarrhea and poor feeding may be seen.

Etiology

The etiological agents of urinary tract infection are listed in Table 60.13.

Table 60.13 Etiological agents of urinary tract infection

Bacteria	Virus	Fungi	Parasites
Gram-negative bacilli • *E. coli* • *Proteus* species • *Klebsiella*	Adenovirus	*Candida albicans*	*Trichomonas vaginalis*

• *Enterobacter* • *Pseudomonas*			
Gram-positive cocci • *Staphylococcus aureus* • *Staphylococcus epidermidis* • *Staphylococcus saprophyticus* • *Enterococcus* species			*Schistosoma haematobium*
Gram-negative cocci • *Neisseria gonorrhoeae*			*Enterobius vermicularis*
Others • *Mycobacterium tuberculosis* • *Salmonella* species • *Gardnerella vaginalis*			

Laboratory Diagnosis

Quantitative cultures are necessary for the diagnosis of urinary tract infection as it differentiates between infection, colonisation and contamination.

Collection and transport of specimens Figure 60.8 summarises the different types of urine samples that can be collected.

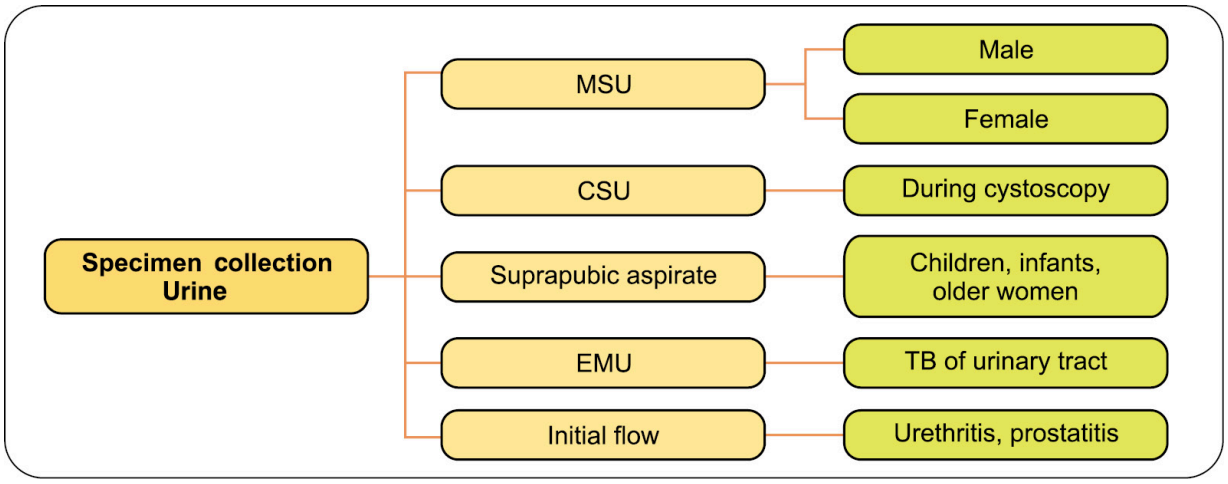


Fig. 60.8 Types of urine samples to be collected (MSU-midstream urine, CSU-catheter sample of urine, EMU–early morning urine)

Collection procedures

Midstream urine (MSU) A midstream urine sample is appropriate for bacterial culture. The patient should be provided with a wide-mouthed, screw-capped, leak-proof, sterile universal container. It is important to give instructions for proper collection. The patient is advised to clean the genital area and to void the first part of the urine into the toilet, then to collect the 'midstream' urine in the container, and finally, to void the last part into the toilet.

Catheter sample urine (CSU) If the patient has been catheterised, the sample is collected as follows: The area over the catheter is first cleaned with alcohol, after donning clean gloves. Then, with the help of a sterile syringe and needle, the urine sample is drawn and put into the universal container. At no time should the urine be collected from the uro-bag or by opening the connecting draining tube.

Suprapubic aspirate If the sample for culture is from an infant, a suprapubic aspirate is collected with a sterile syringe and needle under aseptic precautions by puncturing the suprapubic area.

Early morning urine (EMU) If renal tuberculosis has to be investigated, three urine samples are collected on consecutive days. The first, morning urine sample is collected in entirety.

If pyelonephritis or upper urinary tract infection is suspected, a blood culture is also required. If the site and side of the kidneys must be identified, an invasive sample collected with a catheter from each ureter is required.

Transport

The sample must be transported at room temperature within half an hour or refrigerated at 4°C for up to four hours. Beyond this time, such a sample should not be processed for bacterial culture. For the interpretation of the results, quantitative cultures need to be done. Since urine is a good culture medium, there may be multiplication of contaminating bacteria if the sample is kept outside for a long period, thus increasing bacterial counts and leading to a false positive result.

If the sample is from a patient who has no immediate access to a health care facility, and transport to the facility would exceed four hours, a special

container with 1.8% boric acid is provided. Urine is collected as described above and this can be kept for up to 24 hours.

Approach to Diagnosis of Urinary Tract Infection

Figure 60.9 is an algorithm of the approach to the diagnosis of urinary tract infections.

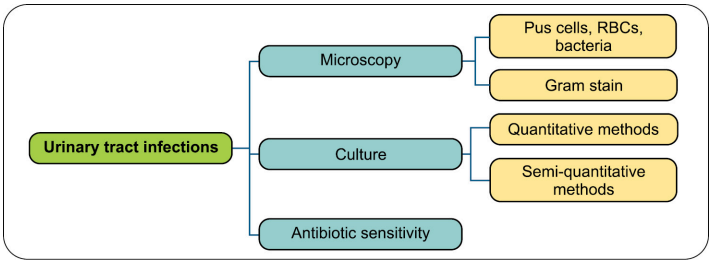


Fig. 60.9 Flowchart of approach to the diagnosis of urinary tract infections

1. Microscopy An uncentrifuged sample is examined by direct microscopy for pus cells and bacteria.

2. Screening Because urinary tract infection is a common problem and bacteriological facilities are not always available, several screening techniques have been introduced for the presumptive diagnosis of significant bacteriuria.

Dipstick tests

i) **Griess nitrite test** is based on the absence of nitrite in normal urine. The presence of nitrite indicates the presence of nitrite-reducing bacteria in urine.

ii) **Leukocyte esterase test** detects the presence of intact or lysed leukocytes.

A positive dipstick test helps in diagnosing cystitis and a negative test rules out cystitis.

Catalase test The presence of catalase as evidenced by frothing on addition of hydrogen peroxide indicates bacteriuria, though a positive result is obtained in hematuria also.

Triphenyl tetrazolium chloride (TTC) test This is based on the production of a pink–red precipitate in the reagent as a result of the respiratory activity of growing bacteria.

Gram stain Microscopic demonstration of bacteria in Gram-stained films of urine is diagnostic.

Glucose test paper This is based on the utilisation of minute amounts of glucose present in normal urine by infection-causing bacteria.

Dip slide culture Agar-coated slides are immersed in urine or even exposed to the stream of urine during voiding, incubated and the growth estimated by colony counting or by colour change of the indicator.

None of the screening methods is as sensitive or reliable as culture.

3. Culture Quantitative culture provides an estimate of the total number of bacteria in 1 mL of urine. However, this is not practically done in the laboratory. So a semi-quantitative method using a standard calibrated loop is done as follows:

Semi-quantitative culturing is done on blood agar and MacConkey agar with a calibrated loop. A colony count of **10^5/mL is considered significant**. Counts between **10^4/mL and 10^5/mL are of doubtful significance**. Counts less than this are considered significant if:

- The patient is already on antibiotics
- There is obstruction in the urinary tract
- A fungal infection is present
- Pyelonephritis is present
- The specimen has been collected by suprapubic aspiration

If ≥ 3 types of organisms are grown, these are considered as contaminants, which may have been introduced into the urine sample from the perineal region or skin or external urethral meatus due to improper collection.

Detection of antibody-coated bacteria differentiates upper UTI (involvement of the kidney) from lower UTI. It is presumed that upper UTI results in antibody response, which allows the antibody to coat the microorganism. This can be detected in the urine by immunofluorescence using antihuman immunoglobulin tagged to a fluorescent dye.

Antibiotic sensitivity testing If culture is suggestive of infection, an antibiotic sensitivity test is done. If a rapid report is needed and direct microscopy suggests significant pyuria, a primary susceptibility test with the urine specimen itself is done to permit initiation of early specific antibiotic treatment. This must be further confirmed by an antibiotic sensitivity test using the bacterium that is recovered in culture.

Treatment

The most suitable method of treatment is with antibiotics that are able to reach high concentrations in the renal parenchyma or urine.

Community onset UTI can be treated with ciprofloxacin and nitrofurantoin (for out-patients).

For complicated UTI and those with renal involvement, a higher antibiotic like third-generation cephalosporin or ceftriaxone, may be given. In case of hospital-acquired UTI and/or catheter-associated UTI, carbapenems or piperacillin/tazobactam may be given. Removal of catheter and catheter care must also be carried out to prevent recurrence.

SEXUALLY TRANSMITTED INFECTIONS

Sexually transmitted infections (STIs) are a group of infectious conditions that are transmitted predominantly or entirely by sexual or close body contact. Bacteria and viruses are the main etiological agents.

Clinical Presentation

Sexually transmitted diseases may present in the following ways:

1. **Vaginal discharge,** which may be:

    - profuse watery discharge, as in bacterial vaginosis;
    - thick white discharge, as in candidosis; or
    - frothy discharge, as in trichomoniasis.

2. **Cervical/urethral discharge** (sometimes vaginal discharge) is due to *Neisseria gonorrhoeae* or *Chlamydia trachomatis*.
3. **Genital ulcer** is caused by herpes simplex virus types 1 and 2 (lesion is called genital herpes), *Treponema pallidum* (chancre), *Haemophilus ducreyi* (chancroid), certain serotypes of *Chlamydia trachomatis* (lymphogranuloma venereum) and *Klebsiella granulomatis* (formerly called *Donovania granulomatis* and *Calymmatobacterium granulomatis*/granuloma inguinale).

The main presenting features are ulceration, pain and lymphadenopathy.

- In **chancre**, a single ulcer is the usual presentation, and is indurated (hard), painless and not tender, with moderate lymph node swelling and no bubo.
- In **genital herpes**, multiple, non-indurated, painful and markedly tender ulcers are seen with moderate-to-no lymph node swelling and no bubo.
- In **chancroid**, single (or multiple), non-indurated (soft), painful and markedly tender ulcer (or ulcers) occur, with marked lymph node swelling and bubo formation.
- **Lymphogranuloma venereum** (LGV) is characterised by the development of transient genital ulcer(s) or papule(s), followed by the appearance of tender inguinal and/or femoral lymphadenopathy with swollen, matted lymph nodes along the course of the inguinal ligament.
- **Donovanosis** produces painless lumps, usually in the genitalia, anal region or groin. The sores are usually painless, but may become painful if secondary bacterial infection occurs.

4. **Pelvic inflammatory disease (PID)** The main presenting features are lower abdominal pain, vaginal discharge and fever. *N. gonorrhoeae* and
C. trachomatis are the principal pathogens.

Etiology

Etiological agents of sexually transmitted infections are listed in Table 60.14.

Table 60.14 Etiology of sexually transmitted infections (STIs)

Bacteria	Virus	Fungi	Parasites
T. pallidum	Herpes simplex virus	*Candida albicans*	*T. vaginalis*
N. gonorrhoeae	Cytomegalovirus		
Chlamydia trachomatis	Human papillomavirus		
Gardnerella vaginalis	Human immunodeficiency virus		
Haemophilus ducreyi	Hepatitis B virus		
Klebsiella granulomatis	*Molluscum contagiosum*		

Approach to Diagnosis of Suspected Sexually Transmitted Infections

Figure 60.10 is an algorithm of the approach to the diagnosis of suspected sexually transmitted diseases.

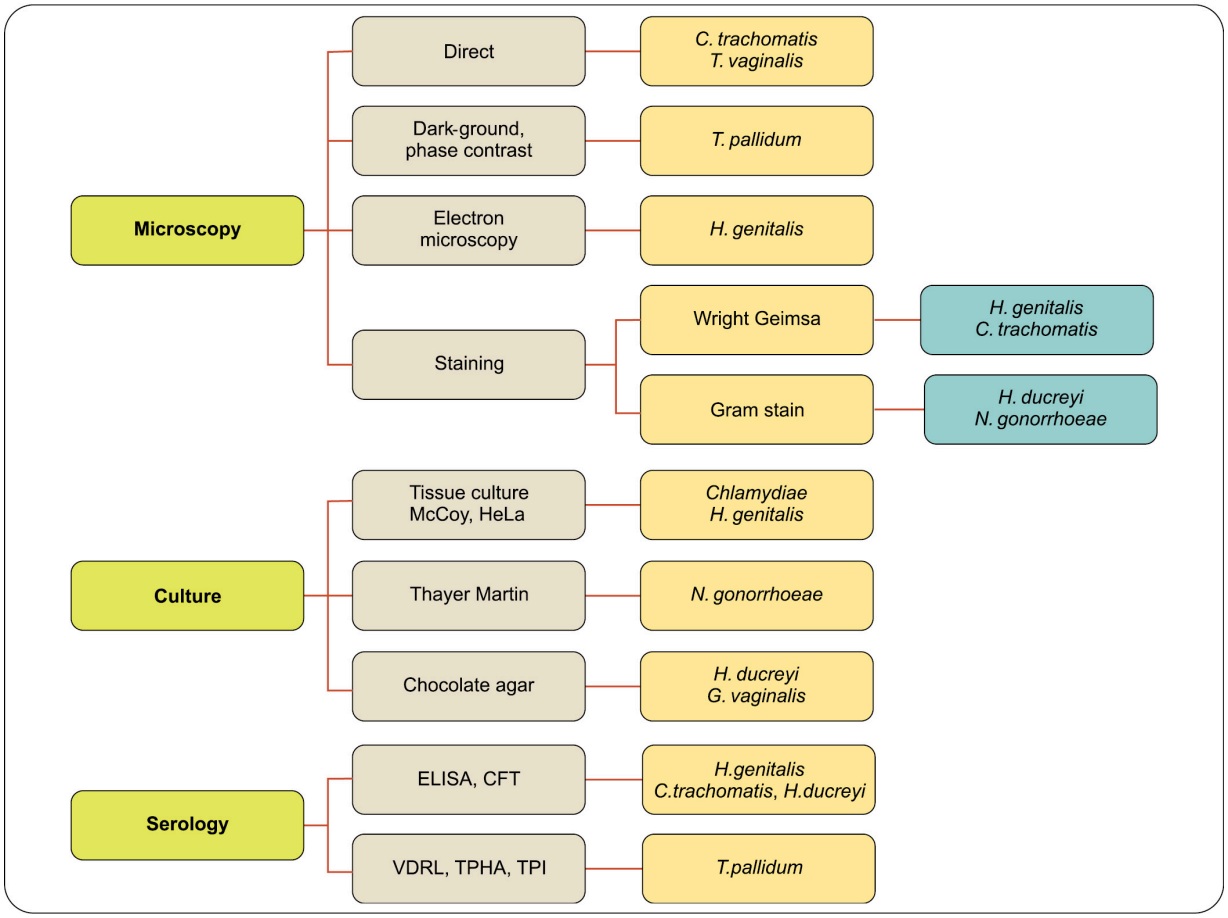


Fig. 60.10 Algorithm of approach to diagnosis of sexually transmitted infections (VDRL—Venereal Disease Research Laboratory test, TPHA—*T. pallidum* hemagglutination assay; TPI—*T. pallidum* immobilisation test)

Collection and Transport of Specimens

Specimen

- Discharge from the infected area/lesion When the vaginal, cervical or urethral discharge is profuse, it can be collected in a sterile container.

- Swabs When the discharge from the infected area is scanty or very thick, it is collected with a sterile swab or endocervical curette from the base and edges of the ulcer.
- Fluid from genital vesicles Sterile capillary tubes are used to collect material from incised genital vesicles.
- Blood This is collected for serological tests and for culture (in suspected gonococcal bacteremia).

Transport The causative organisms are delicate and do not remain viable for long outside the body. Immediate transport to the laboratory and inoculation is necessary. The transport medium used is modified Stuart's medium.

Laboratory Diagnosis

1. Microscopy

- A wet mount is made of vaginal discharge:
    - In **trichomoniasis**, pus cells and motile trophozoites of *Trichomonas vaginalis* are seen.
    - In **candidiasis**, yeast cells and pseudohyphae of *Candida albicans* are noted**.**
- A gram-stained smear is made of the discharge or swabs from a genital ulcer:
    - By examining a gram-stained smear of vaginal discharge, a diagnosis of bacterial vaginosis can be made based on the Nugent score, which takes into consideration the decreased number of lactobacilli (normal flora), increased number of gram-variable pleomorphic coccobacilli (suggestive of *Gardnerella vaginalis*) and the presence of curved gram-negative bacilli (suggestive of *Mobiluncus*).
    - 'Clue cells' are vaginal epithelial cells with adherent bacteria (*G. vaginalis*) that give them a stippled appearance; the presence of such cells and absence of pus cells suggests a diagnosis of bacterial vaginosis.

    - In gonorrhea in women, numerous polymorphs and gram-negative intracellular diplococci are seen in the cervical/urethral discharge, and sometimes in the vaginal discharge.
    - In chancroid due to *Haemophilus ducreyi*, gram-negative coccobacilli are seen in pairs.
    - Gram-positive budding yeast cells can be seen in vaginal infection due to *Candida albicans*.
- On staining with **Giemsa stain**, *Klebsiella granulomatis* (the cause of granuloma inguinale) is usually demonstrated in direct smears of material from lesions on the genitalia, in the groin and on the perineum; the coccoid rod-shaped organisms are found within histiocytes and giant cells.
- In suspected primary or secondary syphilis, **dark-ground microscopy** is performed on preparations of material from the chancre (primary syphilis) or from mucous membranes (secondary syphilis) to demonstrate spirochetes.

2. Culture This is done to isolate some of the organisms listed above:

- Irradiated McCoy cells are used to isolate chlamydiae.
- Thayer Martin medium, among others, is used to isolate*N. gonorrhoeae*.
- Chocolate agar is used to isolate *H. ducreyi* and *G. vaginalis*.
- Cell culture is done for herpes simplex viruses types 1 and 2.
- Sabouraud dextrose agar is used for *Candida albicans*.
- *K. granulomatis* is difficult to cultivate.

3. Serology

- Blood may be collected for culture during suspected gonococcal bacteremia.
- Serum samples are used for serological tests to establish the diagnosis of syphilis (VDRL, TPHA, TPI), chlamydial infection (CFT) and genital herpes infections (ELISA).

- Molecular tests based on nucleic acid amplification tests (NAAT) have now replaced conventional culture for *Chlamydia* and *Mycoplasma* and other organisms that are difficult to cultivate.

Treatment

Treatment guidelines have been developed for the prevention, management and control of reproductive tract infections including sexually transmitted infections (STI) by the Ministry of Health and Family Welfare. It advocates following a syndromic approach to treatment.

1. Tab. Azithromycin 1 g and tab. Cefixime 400 mg for the following:

    - Urethral discharge (UD)
    - Cervical discharge (CD)
    - Ano-rectal discharge (ARD)
    - Painful scrotal swelling (PSS)

2. Tab. Secnidazole 2 g and tab. Fluconazole 150 mg for vaginal discharge
3. Benzathine penicillin, 2.4 million units administered intramuscularly and tab. Azithromycin 1 g for genital ulcer (non-herpetic) or doxycycline for those who are allergic to penicillin
4. Acyclovir for herpetic genital ulcer
5. Doxycycline and azithromycin for bubo

Some specific infections and their treatments are presented in Table 60.15.

Table 60.15 Antibiotics for some of the common STDs

Syphilis	**Benzathine penicillin G**
Gonorrhea	Ceftriaxone, azithromycin
Chlamydial infection	Ceftriaxone (single dose) + Doxycycline (14 days)
Chancroid	Azithromycin
Lymphogranuloma venereum	Doxycycline
Donovanosis	Doxycycline
Genital herpes	Acyclovir, valacyclovir

Some of the non-sexually transmitted infections of the female genital and reproductive tract include:

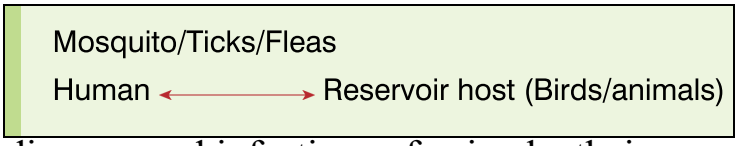
- Vaginosis
- Cervicitis
- Endometritis
- Salpingitis

In males, this includes:

- Prostatitis

VECTOR-BORNE AND ZOONOTIC DISEASES

Vector-borne infections are transmitted by vectors (mosquitoes, ticks and fleas) through bites. These vectors carry infective pathogens such as viruses, bacteria, and protozoa, which can be transferred from one host (carrier) to another in the process of sucking blood. These diseases account for a significant number of human illnesses and deaths each year. Changing ecosystems and environmental degradation have resulted in the rise of vector-borne infections. Some common vector-borne infections and their vectors and reservoirs are shown in Table 60.16.



Zoonoses are diseases and infections of animals; their causative agents are transmitted between animals and humans. This transmission may occur directly (e.g., anthrax) or through a vector (Japanese encephalitis).

Table 60.16 Common vector-borne diseases

Diseases	Vector	Reservoir
Plague	Rat flea	Rats
Rickettsial diseases	Ticks	Deer, rodents
Lyme disease	Ticks	Deer, rodents, opossums

Tularemia	Ticks, biting flies	Rodents
JE	Female mosquito (Culex)	Water birds
West Nile	Female mosquito (Culex)	Wild birds
Dengue	Female mosquito (Culex)	Primates / infected persons with viremia)
Yellow fever	Female mosquito	Primates
Chikungunya	Female mosquito	Primates/birds
Malaria	Female mosquito (Anopheles)	Infected human being
Leishmaniasis	Sandfly	
Filariasis	Female mosquito (Anopheles)	Infected human being
Trypanosomiasis	Tsetse fly	Cattle, horses

Etiology

Zoonotic diseases commonly occur in individuals who handle animals/animal products. The most common zoonotic diseases are listed in Table 60.17.

Zoonotic infections commonly seen in India are:

- Salmonellosis
- Leptospirosis
- Anthrax
- Brucellosis (in certain geographic areas)
- Scrub typhus
- Japanese encephalitis
- Rabies
- Chikungunya
- KFD
- Leishmaniasis

Table 60.17 Common zoonotic diseases

Bacteria	Rickettsia (vector-	Virus	Parasite	Fungus

	borne)			
Anthrax Plague (vector-borne)	Scrub typhus	Rabies	Taeniasis	Zoophilic dermatophytes
Brucellosis	Murine typhus	Yellow fever (vector-borne)	Echinococcosis	
Leptospirosis	Tick typhus	Japanese encephalitis (vector-borne)	Leishmaniasis (vector-borne)	
Salmonellosis	Q fever	KFD (vector-borne)	Toxoplasmosis	
		Chikungunya (vector-borne)		

Laboratory Diagnosis

This is important for the diagnosis of zoonoses. In humans and animals, this is based on:

- Isolation of the causative agent
- Serology
- Autopsy

Collection of specimens Specimens are collected according to the site of the lesion. Tables 60.18, 60.19, 60.20, 60.21 and 60.22 list the types of specimens to be collected and laboratory diagnostic tests for bacterial, rickettsial, viral, parasitic and fungal zoonoses, respectively.

Table 60.18 Laboratory procedures for bacterial zoonoses

Disease	Sample	Microscopy	Culture	Serology	Others
Cutaneous anthrax	Fluid from eschar	Gram-positive bacilli (bamboo stick appearance)	Nutrient agar (Medusa head)	Ascoli's thermoprecipitin test CFT	Lysis by gamma phage
Pulmonary anthrax	Sputum Stool		Blood agar (string of pearls)	ELISA	Direct fluorescent antibody test
Intestinal anthrax		McFadyean's reaction	Gelatin stab (inverted fir tree)		

Acute brucellosis (undulant fever)	Blood	Gram-negative coccobacilli	Castaneda method	Standard agglutination test ELISA, CFT	Skin test
Bubonic plague	Fluid from buboes	Gram-negative bacilli	Nutrient agar	Passive hemagglutination	PCR
Pneumonic plague	Sputum	Bipolar staining	Blood agar		
Septicemic plague	Blood	Safety pin appearance	Ghee broth ('stalactite' growth)		
Salmonellosis	Stool Food	Gram-negative bacilli	MacConkey agar Wilson and Blair medium	Widal test	
Leptospirosis	Blood Urine	Dark-ground microscope; spirochete	Korthof's medium Stuart's medium Fletcher's medium	Microscopic agglutination test	
Tuberculosis (*M. bovis*)	Sputum	Acid-fast bacilli	LJ medium		PCR

Table 60.19 Laboratory procedures for rickettsial zoonoses

Disease	Sample	Culture	Serology
Scrub typhus	Blood	Yolk sac of chick embryo	Weil–Felix test
Murine typhus	Blood	Yolk sac of chick embryo	Weil–Felix test
Tick	Blood	Yolk sac of chick embryo	CFT

Table 60.20 Laboratory procedures for viral zoonoses

Disease	Sample	Microscopy	Culture	Serology/ molecular detection
Rabies	Ante-mortem— corneal impression smear Skin biopsy; saliva	Immuno-fluorescence and immunoperoxidase staining Negri bodies	Tissue culture (WI 38, BHK 21)	Rabies virus neutralisation test to detect antibodies in CSF Reverse transcriptase

Disease	Sample	Microscopy	Culture	Serology/ molecular detection
	Post-mortem—brain			PCR
Yellow fever	Blood	Not applicable	Embryonated eggs and tissue culture	Hemagglutination inhibition
Japanese encephalitis	CSF	Not applicable	Intracerebral inoculation into suckling mice	CFT Neutralisation
KFD		Not applicable	Tissue culture	Immuno fluorescence
Chikungunya		Not applicable	Human embryonic lung tissue and Vero cell lines	ELISA IgM antibodies capture ELSA (MAC-ELISA) and molecular diagnosis by nested PCR and real-time PCR

Table 60.21 Laboratory procedures for parasitic zoonoses

Disease	Sample	Microscopy	Serology
Taenia	Stool	Egg	ELISA
Echinococcus	Stool	Hydatid cysts with scolices from biopsies or after surgical removal	ELISA Hemagglutination Indirect fluorescent antibody
Leishmania	Blood	Peripheral blood, amastigote (LD bodies)	Aldehyde test Antimony test CFT

Table 60.22 Laboratory procedures for fungal zoonoses

Microscopy	Disease	Sample	Culture
	Zoophilic dermatophytes		
KOH preparation	*Microsporum canis*	Skin scraping	SDA
	Trichophyton verrucosum	Hair clipping	
	T. equinum	Nail clipping	

MISCELLANEOUS INFECTIONS

EYE INFECTIONS

Eye infections commonly present as conjunctivitis, corneal ulcer or endophthalmitis. Infections may involve the external parts of the eye (ocular adnexa) or may be intraocular.

Infections of the Ocular Adnexa

- Conjunctivitis—caused by bacteria (*Chlamydia*, *Haemophilus influenzae*, *S. pneumoniae* and *S. aureus*), viruses (adenoenterovirus) and parasites (Toxoplasma, microsporidia).
- Keratitis—a common ocular infection caused by bacteria, fungi, viruses like herpes simplex zoster; parasites like free-living protozoa such as Acanthamoeba can cause corneal infections.
- Orbital cellulitis—commonly seen in children.
- Trachoma—chronic keratoconjunctivitis caused by *Chlamydia trachomatis*.
- Ophthalmia neonatorum is a conjunctival inflammation occurring during the first month of life. It is caused by bacteria including *Chlamydia*
  (*N. gonorrhoeae* infection can cause corneal ulceration) and virus (herpes simplex). There is severe purulent conjunctivitis. Gram stain and culture can confirm bacterial etiology.

Intraocular Infections

Endophthalmitis, although not very common, can be devastating. It occurs following a penetrating injury to the eye (exogenous), leading to panophthalmitis or as a complication of a bloodstream (endogenous) infection. Almost any organism including bacteria and fungi can cause endophthalmitis.

Laboratory Diagnosis

Diagnosis is made by smear examination of the eye discharge, swabs or

aspirates. Culture often yields the causative bacterial agent. As the samples are very minimal, a bedside smear and inoculation on culture media often gives better results.

Treatment

Topical antibiotics, ciprofloxacin, azithromycin and vancomycin drops, in conjunction with systemic therapy are required in endophthalmitis and other ocular infections.

In herpes infection, acyclovir together with topical therapy of acyclovir eye ointment is required.

EAR INFECTIONS

Infections of the ear may be seen in the external ear or middle ear. They may involve the skin, cartilage, mastoid cavity, periosteum or the tympanic membrane. The organisms involved are bacteria, viruses and fungi. Some of the clinical entities are:

- Auricular cellulitis—involves the skin over the external ear due to minor trauma and is commonly caused by *S. aureus* and streptococci. Warm compress and first-generation cephalosporin (cefazolin) are used to treat mild infection. Severe infection may require IV beta-lactamase-inhibiting antibiotics.
- Perchondritis—an infection of the perichondrium of the auricular cartilage is caused by trauma to the cartilage (e.g., ear piercing and laceration). The most common organisms involved are *S. aureus* and *P. aeruginosa*. Diagnosis may be confirmed by drainage and culture of the pus. Treatment is by quinolones, antipseudomonal penicillin (piperacillin) or cephalosporin if staphylococci are isolated.
- External otitis—involves the auditory meatus and is caused by the accumulation of moisture and macerated desquamated epithelium of external ear. Acute external otitis infection is caused by *S. aureus* or
  P. aeruginosa commonly. Furuncle at the junction of the cartilage and the meatus with hair follicles may require incision and

drainage followed by antipseudomonal treatment (piperacillin) or cefazolin for staphylococcal infection. Diffuse otitis externa is known as swimmer's ear. A chronic form of the infection may occur due to constant irritation caused by drainage from the middle ear. Invasive otitis externa called malignant or necrotising otitis externa may be seen in diabetics or immunocompromised individuals. This is a potentially life-threatening and aggressive infection. If left untreated, it may progress to the base of the skull (skull base osteomyelitis) and may involve the meninges and the brain. Organisms responsible are *Pseudomonas*, staphylococci, *Aspergillus* and *Actinomyces*. Treatment is based on the etiological agents, either organism-specific broad-spectrum antibiotics like piperacillin, tazobactam, fluoroquinolones and aminoglycosides or antifungal amphotericin.

- Otitis media—middle ear infection may be acute (acute otitis media) or chronic acute infection following a viral throat or rhinosinusitis infection (RSV, influenza, rhinovirus infections) or bacterial infection (*S. pneumoniae*, *H. influenzae*-non-typeable, and *M. catarrhalis* and MRSA). Clinical features include painful discharge due to fluid accumulation in the middle ear. This is accompanied by diminished hearing and fever. There may be vertigo and nystagmus. Treatment is by antibiotics like amoxycillin–clavulanic acid or piperacillin–tazobactam or as per the sensitivity of the isolated pathogen from ear swab or discharge. Chronic otitis media is characterised by persistent infection with conductive hearing loss and otorrhea. There may be perforation of the tympanic membrane.
- Mastoiditis—this is an infection in the mastoid air cells, which leads to the erosion of the adjoining bone and periosteal abscess through the mastoid tip to deep neck abscess. Broad empiric treatment is initiated followed by pus drainage. This condition is treated with antibiotics specific to the organism which could be *Pseudomonas, Staphylococcus, Pneumococcus* and *Haemophilus.*

CONGENITAL INFECTIONS

Infections acquired by the fetus during gestation are referred to as congenital infections. These are acquired from the mother who may have the active infection or latent form of the infection. The infective organisms cross the placental barrier and enter the bloodstream of the fetus. If the infection is acquired by the fetus in the early stage of development and organogenesis, the pregnancy generally ends in abortion or the baby is born with major congenital defects. If the infection is acquired in the later trimesters, the pregnancy may go to term and the baby may manifest the disease during the early or late neonatal period. Bacteria, viruses and parasites may cause congenital infections (Table 60.23).

Antenatal diagnosis may be made by scanning and by the demonstration of antibodies in the mother or by amniocentesis.

Postnatal diagnosis is made by detecting specific IgM to the specific infectious agent in the blood of the neonate (as it is produced in the fetus in response to the infection) by immunochromatography or ELISA.

Culture may be done from blood (in case of neonatal septicemia), CSF (meningitis) or other appropriate samples.

Viral agents may be detected by nucleic acid amplification techniques from the blood or tissues.

Table 60.23 Common microorganisms associated with congenital infections

Bacteria	Virus	Parasites
Treponema pallidum	CMV	*Toxoplasma*
Streptococcus agalactiae	Rubella	*Plasmodium*
Listeria	HSV	
Mycobacteria	Parvovirus 19	
Gram-negative enteric pathogens	Hepatitis B and C	
	HIV	
	Papillomavirus	

Treatment

In case of bacterial infections, specific antibiotics are given in pediatric doses. HIV is detected by detecting proviral DNA and treating with zidovudine, nevirapine and lamivudine. Such patients have to be treated for life.

Toxoplasmosis is treated with pyrimethamine and sulphadiazine.

Prevention of Congenital Infection

All pregnant women are screened for common infections and treated adequately to prevent congenital infections. They are also vaccinated against tetanus.

Questions

Case Studies

1. A 6-year-old girl presented with a 24-hour history of fever, vomiting, and complaints of sore throat. On physical examination she had a temperature of 38.5°C, her tonsillar region appeared inflamed and was covered with a dirty gray patch with exudates. She had several enlarged cervical lymph nodes.

a) What is your provisional diagnosis?
b) What laboratory tests will you do to arrive at an etiological diagnosis?
c) List two other bacterial infections that can present with a similar clinical picture.
d) How can children be protected from this infection?

2. A 19-year-old female complained of left flank pain, fevers, and chills with increased urinary frequency. The urine was foul-smelling. On examination of the urine, there were >50 leucocytes per high-power field.

a) Name four common organisms associated with urinary tract infection in an adult female.
b) Name 3 rapid tests you can do to confirm the diagnosis.
c) Describe briefly the method of semi-quantitative culture of urine.
d) How will you decide on specific antibiotic therapy for this patient?

3. A 20-year-old migrant labourer presented to the OPD with painful genital lesions. He gave a history of sexual contact with a commercial sex worker 10 days ago.

a) What is the differential diagnosis of ulcerative genital lesions?
b) Which rapid test can be done so that specific therapy could be started?
c) Describe the method of confirming the diagnosis by laboratory tests.
d) Mention the antibiotic resistance of this organism.

4. An 18-year-old male presented to the OPD with history of diarrhea and abdominal discomfort. He gave a history of having eaten street food. On examination, the patient was afebrile and had normal vital signs. The abdominal examination was notable for mild lower abdominal tenderness. Microscopic examination of feces demonstrated watery stool that was negative for occult blood. There were plenty of pus cells with no ova or cysts.

a) How will you confirm the etiology of this diarrhea?
b) List five agents causing infective diarrhea.
c) Give an example of toxin-mediated diarrhea and describe its mechanism of action.
d) Discuss the role of antibiotics causing diarrhea.

5. A chronic alcoholic adult male was admitted to the hospital with pain in the right hypochondrium, loss of appetite and vomiting. He gave a history of having had blood transfusion 3 years ago. His serum alanine aminotransferase (ALT) level was 220 U/L (normal, <35), and his aspartate transaminase (AST) level was 140 U/L (normal, <40).

a) List the common infective causes of this condition.

b) What serological test(s) will you do to arrive at a diagnosis?
c) How will you monitor the progression of this infection?
d) What is the effective vaccine available to prevent this infection?

6. A two-year-old male child was brought to the emergency department with high-grade fever, vomiting and convulsions. On examination, the temperature was 101°F, respiratory rate was 40 per minute and shallow and the child was irritable.

a) What is the most probable diagnosis?
b) Mention a rapid bedside test to diagnose this condition.
c) List the common agents associated with this infection in a child.
d) How can children be protected from this infection?

Short Answers

1. Five causes of acute pyogenic meningitis
2. Laboratory diagnosis of UTI
3. Common zoonotic infections in India
4. Pathogenesis of cellulitis
5. Mechanism of one toxin-mediated food poisoning
6. Non-gonococcal urethritis

Part VI: Clinical Microbiology as Applied to Infectious Diseases

CHAPTER 61

Emerging and Re-emerging Infections

Chapter navigation

- Bioterrorism
- National programmes for disease detection and reporting

INTRODUCTION

Infectious diseases continue to be one of the leading causes of death worldwide. Even as infections like smallpox and polio have been eradicated, the emergence of new infectious diseases and re-emergence of old infections continue to be a cause for concern. Humans, domestic animals and wildlife are closely linked in the epidemiology of infectious diseases. Human-induced environmental changes, interspecies contact, altered social conditions, demography and medical technology affect the survival of microbes. Any change in this delicate balance of hosts, parasites and ecosystems causes new infections to emerge or old diseases to re-emerge.

Emerging infectious diseases These are diseases that have appeared for the first time in a population or that did not exist previously in an area but are rapidly increasing in incidence in that geographical range.

The factors that contribute to emerging infections are presented in Fig. 61.1.

Re-emerging infections These are infections that caused recognised health problems in the past before their incidence dropped to such low levels that they were no longer considered public health problems but which are again showing an upward trend in incidence or prevalence in that geographic area

or worldwide.

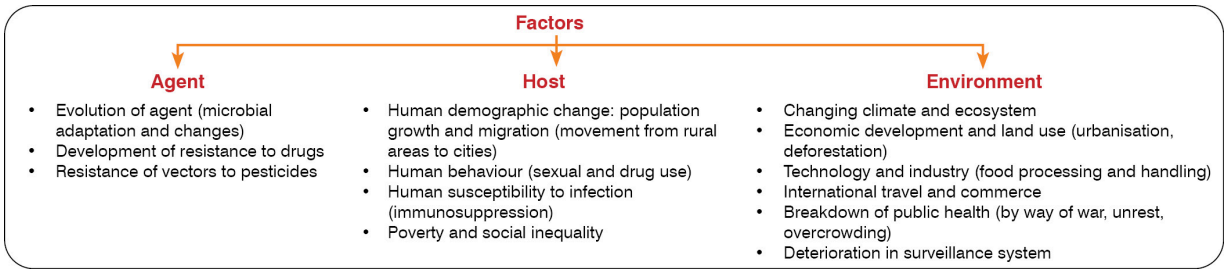

Factors
Agent
Evolution of agent (microbial adaptation and changes)
Development of resistance to drugs
Resistance of vectors to pesticides
Host
Human demographic change: population growth and migration (movement from rural areas to cities)
Human behaviour (sexual and drug use)
Human susceptibility to infection (immunosuppression)
Poverty and social inequality
Environment
Changing climate and ecosystem
Economic development and land use (urbanisation, deforestation)
Technology and industry (food processing and handling)
International travel and commerce
Breakdown of public health (by way of war, unrest, overcrowding)
Deterioration in surveillance system

Fig. 61.1 Factors contributing to emerging infections

DISEASE TRANSMISSION FROM ANIMALS TO HUMANS

About two-thirds of emerging infections originate from animals—both wild and domestic—and, therefore, are **zoonotic**.

Animals displaced from their original habitats due to deforestation or climate change and those that migrate in search of food have been cited as a possible cause of Lassa fever.

Human penetration in unpopulated areas brings them closer to animal reservoirs or vectors. This may have contributed to the development of organisms causing diseases like yellow fever and malaria.

Deforestation forces animals and human into contact. An example of this is Kyasanur Forest Disease (KFD) in the Shimoga district of Karnataka, which has now spread to the adjoining state, Kerala.

Global warming has fueled the spread of malaria, dengue, leishmaniasis and filariasis.

Poverty and malnutrition have contributed to a severe infection–disease cycle.

Changes in agricultural and food production patterns may have led to the development of agents of food-borne infections such as *E. coli.*

Increased international travel and outdoor activity may also have led to closer contact between humans and animals, leading to the emergence of pathogens like MERS-CoV.

Table 61.1 lists the new infectious diseases identified since 1997.

Table 61.1 New infectious diseases recognised since 1997

Year	Agent	Disease

1997	H5N1	Avian flu (bird flu)
1999	*Nipah* virus (Paramyxoviridae, genus *Henipavirus*)	Encephalitis and respiratory illness in Malaysia and Singapore
2003	Coronavirus	SARS-CoV
2009	H1N1	Pandemic (H1N1) influenza
2011	CCHF	Crimean Congo hemorrhagic fever outbreak reported in Gujarat
2012	MERS-CoV	Middle East respiratory syndrome caused by a coronavirus—first identified in Saudi Arabia
2013	H7N9 Influenza	A novel reassortant avian-origin influenza A (H7N9) virus isolated from respiratory secretions of three critically ill patients from Shanghai, China, in March 2013
2013	Ebola virus (re-emerged)	A total of 28,616 cases with 11,310 deaths were reported in Guinea, Liberia, and Sierra Leone (2013–2016)
2016	Zika virus	First appeared in Brazil and spread to other parts of South and North America
2017	Monkeypox	Nigeria reported a large outbreak; since 1970, human cases of monkeypox have been reported from 10 African countries
2018	Nipah virus	Originated first in Malaysia in 1999, then reached Bangladesh and eastern parts of India
2019	COVID-19 (SARS-CoV-2)	Originated in the Wuhan district of China in December 2019; started spreading to other countries by January 2020 and was declared a pandemic in March 2020

PUBLIC HEALTH EMERGENCY INFECTIONS

The World Health Organization has prioritised the following diseases for public health emergency and research:

- Crimean–Congo hemorrhagic fever (CCHF)
- Ebola virus disease and Marburg virus disease
- Lassa fever
- Middle East respiratory syndrome coronavirus (MERS-CoV)
- Severe acute respiratory syndrome (SARS)
- Nipah and henipaviral diseases

- Rift Valley fever (RVF)
- Zika
- COVID-19

Zika Virus Disease

WHO declared Zika virus disease as a public health emergency of international concern in February 2016.

This is a viral infection transmitted through the bite of the *Aedes* mosquito (*A. aegypti, A. albopictus*) and is related to dengue, yellow fever and the West Nile virus. Though identified in 1947 in the Zika forest of Uganda, its presence was recognised only recently (in 2015) as a 'mystery disease' when it was reported from Brazil. There was concern in the developed world because of the threat of its spread to the western hemisphere. If the index of suspicion is not high, it is difficult to detect. The symptoms are not specific and resemble symptoms of other viral fevers like fever, headache, joint pains, rash and bloodshot eyes. This infection is a cause for concern as it can be passed from a pregnant mother to the fetus and can cause some birth defects, especially microcephaly. At present, pregnant women are advised not to travel to areas where the Zika virus infection has been reported. No vaccine or antiviral drug is available as yet.

INDIAN SCENARIO OF VARIOUS INFECTIONS

The bacterial and viral infections pertaining to the Indian scenario are given in Table 61.2.

Table 61.2 Emerging and re-emerging infections in India

Bacterial infections	Viral infections
• Plague • Leptospirosis • Brucellosis • Anthrax • Cholera • Scrub typhus	• Influenza-H1N1, avian influenza • Chikungunya • Chandipura • Dengue • Japanese encephalitis • SARS coronavirus, hantavirus, human enterovirus 71 • Crimean Congo hemorrhagic fever (CCHF) • Hantavirus

	• Zika • Nipah • COVID-19

In India, Zika virus was first reported in three cases from the Bapunagar area of Ahmedabad, Gujarat, in January 2017. These cases tested positive for the virus by RT-PCR. This was later confirmed by the WHO. All three cases recovered. The low level of transmission of Zika virus may lead to new cases in the future. Therefore, there is a need to strengthen surveillance. Zika virus antibodies have been detected in the Indian population, indicating protective immunity.

In Gujarat, **Crimean–Congo hemorrhagic fever (CCHF)** was reported for the first time in 2011. It was reported as zoonotic in origin with ticks as the vectors. The outbreak was caused by person-to-person transmission in hospitals.

Nipah virus infection outbreaks This is a food-borne disease that is transmitted by the ingestion of dates contaminated with the urine or saliva of infected bats, following which, person-to-person transmission occurs. This disease originated in Malaysia in 1999 and then reached Bangladesh and the eastern parts of India including Siliguri in West Bengal in 2001. The outbreak started from one hospital and spread to 3 others. Most of the patients were hospital staff or those who had visited patients in one of the four hospitals. The infection spread through the respiratory secretions and urine of infected persons which contained viable viruses. In July 2018, there was an outbreak of Nipah virus that lead to 17 deaths in Kerala. The outbreak was localised to two districts in the state: Kozhikode and Malappuram. As no new cases have been reported since June 2018, the disease has been declared contained.

Antibodies to the viral antigen can be demonstrated by ELISA. RT-PCR is used confirm the viral etiology.

Human hantavirus infection was confirmed for the first time in India in 2000. These cases were initially suspected to be leptospirosis. Antibodies to the human hantavirus were later demonstrated in populations in South India by an ELISA test. In 2005, following the floods in Mumbai, some cases were found to have IgM antibodies to the virus. It may be that the disease exists in India but its incidence is under-reported due to the lack of

awareness.

Early suspicion and rapid confirmation of diagnosis are vital in preventing an emerging infection. Availability of simple, easy-to-perform and affordable tests are a basic necessity for proper management. Rapid control measures, which include contact tracing, quarantine and active surveillance, are required to contain the infection and prevent large-scale outbreaks. Mass vaccination, if available, has to be instituted.

DRUG RESISTANCE

The number of drug-resistant bacteria has increased in the last decade. The main cause of the current crisis in antimicrobial resistance is the uncontrolled and inappropriate use of antibiotics in both industrialised and developing countries. Of major public health concern are the following:

- Multidrug-resistant tuberculosis (MDR and XDR-TB)
- Methicillin-resistant *S. aureus* (MRSA)
- Penicillinase-producing *N. gonorrhoeae* (PPNG)
- Vancomycin-resistant enterococci (VRE)
- Extended-spectrum beta-lactamase (ESBL)-producing gram-negative bacteria
- Carbapenemase-producing enterobacteriaceae (CRE)

Control of Emerging Antimicrobial Resistance

Besides rational use of antimicrobial agents, infection-control measures in hospitals and control of prescription are needed. Creating awareness among the public and restriction of over-the-counter sale of the anti-infective drugs are major drives to control the misuse of antibiotics. Antibiotics should be dispensed exclusively on prescription. Price and quality control of the available drugs requires to be monitored by regulatory authorities.

BIOTERRORISM

Definition Bioterrorism, as defined by the Centers for Disease Control and Prevention, USA, is the deliberate release, by attackers, of an agent that causes one or more of a variety of different diseases.

Categories Based on the risk to national security, these agents have been categorised as A, B and C (Table 61.3). These disease agents exist in nature (with the exception of smallpox, which has been eradicated in the wild) but could be manipulated to make them more dangerous.

Table 61.3 Categories of bioterrorism agents

Category	Property	Examples
Category A	High risk; pose a threat to national security; have the ability to cause social disruption	Anthrax, botulism (via botulinum toxin, which is not transmitted from person to person), plague, smallpox, tularemia, and a collection of viruses that cause hemorrhagic fevers, such as Ebola, Marburg, Lassa and Machupo
Category B	Moderately easy to disseminate and result in low mortality	These include brucellosis, glanders, Q fever and ricin toxin
Category C	Agents that can be engineered for mass dissemination	Nipah virus

COMMUNICABLE DISEASE PROGRAMMES UNDER THE NATIONAL HEALTH MISSION

The major communicable disease programmes in India include the following:

1. Revised National TB Control Programme [Renamed as National Tuberculosis Elimination Programme (NTEP)]

The basic principle of the RNTCP (now called NTEP) is "universal access for quality diagnosis and treatment for all TB patients in the community" with a target of **"reaching the unreached".**

- Testing and screening for pulmonary TB
  - Any **person with symptoms and signs suggestive of TB,** including cough >2 weeks, fever >2 weeks, significant weight loss, hemoptysis, etc., and any abnormality in the chest radiograph must be evaluated for TB.

    - Children with persistent fever and/or cough >2 weeks, loss of weight/no weight gain, and/or contact with pulmonary TB cases must be evaluated for TB.
- Microbiological confirmation on sputum
    - All patients (adults, adolescents and children who are capable of producing sputum) with presumptive pulmonary TB should undergo a quality-assured sputum test for the rapid diagnosis of TB (with AFB smear of at least two samples of sputum, including one early morning sample) for microbiological confirmation.

2. National Leprosy Eradication Programme (NLEP)

The National Leprosy Eradication Programme is one of the communicable diseases programmes of the Govt. of India. The programme includes house-to-house survey to detect new cases and monitor old cases. The strategies adopted in the programme are:

- Early detection through active surveillance by trained health workers
- Regular treatment of cases by setting up multi-drug therapy (MDT) centres in villages around moderate-to-low endemic areas/districts
- Intensified health education and public awareness campaigns to remove social stigma attached to the disease
- Appropriate medical rehabilitation and leprosy ulcer care services

3. National Vector-Borne Disease Control Programme (NVDCP)

This programme was launched in 2003–04 to control vector-borne diseases. It is directed at the control of six vector-borne diseases, which are as follows:

- Malaria
- Filaria

- Kala azar
- Japanese B encephalitis
- Dengue/DHF
- Chikungunya

4. National AIDS Control Programme (NACP)

The objective of this programme is to reduce new infections by 50% and provide comprehensive care and support to all persons living with HIV/AIDS and treatment services for all those who require it.

The programme was initiated in 1992 by the Govt. of India to detect, treat and control HIV infections. It has been a successful programme which now provides testing, treatment and prevention services throughout the country. It is now in its 4th phase (NACP IV). HIV was brought down by the following measures:

- Detection of HIV by counselling and testing in Integrated Counselling and Testing Centres (ICTC)
- Surveillance by testing antenatal women and population at risk
- Early infant diagnosis
- Prevention and care, support and treatment of people living with HIV(PLHIV)

5. Integrated Disease Surveillance Project (IDSP)

Under this programme, weekly disease surveillance data on epidemic-prone diseases are being collected from reporting units such as subcentres, primary health centres, community health centres, hospitals including government and private sector hospitals and medical colleges. District laboratories are being strengthened for the diagnosis of epidemics.

One of the most important objectives of the IDSP is strengthening of the disease surveillance system for epidemic-prone diseases in order to detect and respond to outbreaks. The IDSP receives disease outbreak reports from all states and union territories on a weekly basis through its IDSP portal.

Surveillance is done at the regional, national and global levels. This includes:

- Epidemiological surveillance
- Laboratory surveillance, e.g., influenza surveillance programme
- Ecological surveillance
- Anthropological surveillance
- Investigation and early control measures
- Monitoring and evaluation under the supervision of the Global Outbreak Alert and Response Network (GOARN) for the containment of these infections

Disease detection and reporting

Disease surveillance is recognised as an important tool for the early detection of outbreaks and for instituting effective control measures in a timely manner. Information and communication technology is being used to transmit surveillance data from peripheral laboratories to central agencies.

6. National Immunisation Programmes

- EIP (Expanded Immunisation Programme)
- Pulse Polio programme

7. National Viral Hepatitis Programme

In order to tackle the increasing incidence of viral hepatitis in the country, the Government of India, through the National Centre for Disease Control, has launched the National Viral Hepatitis Surveillance Programme.

The objective of the programme is to:

- Establish a laboratory network for the surveillance of viral hepatitis
- Determine the prevalence of different types of viral hepatitis
- Provide laboratory support for outbreak investigation of hepatitis through an established network of laboratories
- Generate awareness among health care providers and in the community about waterborne and blood-borne hepatitis by creating information and education materials

Key points

- Infectious diseases continue to be among the leading causes of death worldwide due to the emergence of new infectious diseases, re-emergence of old infectious diseases and persistence of intractable infectious diseases. Climatic change, deforestation, etc., contribute significantly to these diseases.
- Most of these infections are zoonotic.
- In India, emerging viral infections include influenza, chikungunya, chandipura, dengue, Japanese encephalitis, SARS coronavirus and CCHF. Emerging bacterial infections include plague, leptospirosis, brucellosis and cholera.
- Surveillance and early control measures help in the containment of these infections.
- Zika virus infection is transmitted through the bite of the Aedes mosquito (*A. aegypti, A. albopictus*) and is related to dengue, yellow fever and West Nile fever and was declared as a public health emergency of international concern by WHO in February 2016.
- National programmes to prevent and control communicable diseases include the diseases TB, HIV, leprosy, malaria, filariasis and the vector-borne diseases–dengue, chikungunya and Japanese encephalitis.

Questions

Short Answers

1. Emerging infections
2. Re-emerging infections in India
3. Strategies to control antibiotic resistance
4. Integrated Disease Surveillance Programme (IDSP)

Part VI: Clinical Microbiology as Applied to Infectious Diseases

Chapter 62

Health Care-Associated Infections

Chapter navigation

- Health care-associated infections
- Laboratory-acquired infections

INTRODUCTION

Approximately 5–10 per cent of patients admitted to health care centres in developed countries, and more than 25 per cent of such patients in developing countries acquire **health care-associated infections (HCAI)**. Such infections increase the burden of morbidity, mortality and costs to the patient and the hospital. It is believed that up to 20 per cent of health care-associated infections in developed countries and 40 per cent in developing countries can be prevented. Moreover, in developed countries, 5–10 per cent of such infections occur in an epidemic or cluster pattern; the percentage is probably higher in developing countries. This has led to concerted efforts to implement infection control programmes in hospitals and health centres.

Health care-associated infections (HCAI) are defined as infections that occur after 48 hours of admission/procedure in the hospital and up to 30 days after procedure/discharge from the hospital. Infections contracted in the hospital but not clinically evident until after discharge also form a part of this spectrum. If a patient is in the incubation period of an infection while in the community but manifests symptoms in the hospital, such an infection will qualify as community-acquired.

In the past, these infections were called hospital-acquired infections

(HAI). However, since it is now recognised that such infections may be acquired at any facility that offers health care and not just hopitals, the name has been changed to health care-associated infections.

Infection control programmes have become a part of patient care and management in almost all hospitals in the developed nations and are fast catching up in the developing countries as governments and managements realise their importance.

Iatrogenic infection This is an infection that results from treatments or investigative procedures; it is not included in the concept of health care-associated infection, although both topics may be dealt with simultaneously.

Laboratory-acquired infections Most hospitals have laboratory services as a part of the health care delivery system. The term laboratory-acquired infections refers to all infections acquired by the personnel working in, or having access to the laboratory through their laboratory-related activities.

There are four biosafety levels.

- **Biosafety level 1** laboratories work with microorganisms that do not cause infection in healthy individuals.
- **Biosafety level 2** laboratories work with organisms that cause infection but do not produce substantial aerosols. They infect the individual but do not spread widely into the community. Infection occurs by autoinoculation, ingestion or exposure to mucous membrane.

  Basic diagnostic microbiology laboratories in hospitals maintain biosafety levels 1 and 2. The potential hazard levels are low and the infectious materials are handled in biological safety cabinets, posing minimal risk to the technical staff.
- **Biosafety level 3** laboratories deal with hazardous materials that pose a significant risk of laboratory-acquired infections to the workers and that may spread to nearby locations. These laboratories have access control and special ventilation systems to remove aerosols.
- Maximum containment laboratories with **biosafety level 4** are highly specialised areas to deal with extremely hazardous material of significant risk to the worker and the community, e.g.,

organisms of bioterrorism. Restricted access, airlocks, sealed openings, change of clothes, shower rooms and decontamination of exhaust air are essential components of these laboratories. There are few such laboratories in the country. They are found at the state or national levels.

Categories of organisms and biosafety level laboratories (BSL)

- BSL 1 – Work on the specimen can be done on open benches, e.g., commensal bacteria like *E. coli* and coagulase-negative staphylococci
- BSL 2 – The specimen requires biosafety cabinets (biosafety cabinet class I or II); the organisms handled in these laboratories are potential pathogens, e.g., salmonella, hepatitis, herpes
- BSL 3 – Organisms that are processed are *Mycobacterium tuberculosis, Brucella, Bacillus anthracis* (processed in biosafety cabinet class II)
- BSL 4 – Organisms like Ebola, Marburg and Nipah viruses are handled in these laboratories

Interplay of three factors in HCAI

Host (patient risk factors)

Microbes	Environment
Multidrug resistance	Unclean, unsterile instruments
Ability to survive in adverse conditions	Health care personnel
Resistance to disinfectants	Improper biomedical waste disposal

COMMON TYPES OF HEALTH CARE-ASSOCIATED INFECTIONS

The exact distribution of such infections in India and other developing countries is unclear. In the developed world, they commonly manifest as infections of the urinary tract, surgical and non-surgical wounds, lungs and respiratory tract, blood (bacteremia) and gastrointestinal tract; tetanus and hepatitis B infection are also noted. The risk factors for some of these infections, the common infecting organisms and methods of preventing

such infections are outlined in the subsequent discussion and in Table 62.1.

Table 62.1 Common types of health care-associated infections, their risk factor and organisms associated

Type of Infection	Risk factor	Common organisms	Method of prevention
Bloodstream infection CRBSI CLABSI	Central or peripheral lines, (femoral, subclavian and jugular) Immunocompromised total parenteral nutrition, end-stage renal disease	ESKAPE pathogens, namely, *E. coli,* staphylococci (MRSA), *Klebsiella,* *Acinetobacter,* *Pseudomonas* *Enterobacter* Other organisms are: *Enterococcus,* *Candida*	Proper preparation of the insertion site, care of IV lines
Pneumonia (may be ventilator-associated)	On ventilator, or tracheostomy	*Acinetobacter* *Pseudomonas* *Klebsiella*	Ventilator care Respiratory physiotherapy
UTI	Catheter	*E. coli,* *Enterococcus*	Catheter care, appropriate types of catheters for long-term use
Surgical site infection	Prolonged surgery, surgical drains	*E. coli,* MRSA	Surgical prophylaxis, proper wound care and dressing
Post-antibiotic diarrhea	Prolonged antibiotic therapy	*Clostridium difficile*	Short duration of therapy (antibiotic monitoring) Probiotics

Health Care-Associated Bacteremia [Bloodstream Infections (BSI)]

These may be the consequences of infections at any site but are commonly caused by infected intravenous cannulae such as central venous catheter and

peripheral lines. The longer the cannula is kept in situ, the greater the risk of infection.

Centers for Disease Control and Prevention (CDC) has defined CRBSI and CLABSI as follows:

1. **Catheter-related bloodstream infection (CRBSI)** Defining a **c**atheter-related bloodstream infection (CRBSI) helps to diagnose and treat a patient appropriately. In order to call it CRBSI, the following criteria must be fulfilled:
   i) Isolation of the same pathogen from a quantitative blood culture drawn through the central line and a second sample from a peripheral vein, where the colony count is three times higher in the sample from the central line as compared to that obtained from peripheral vein
   ii) A shorter time to positive culture (less than 2 hours) in the central line sample than the peripheral sample [differential time to positivity (DTP)]

If the sample collected from the peripheral vein grows an organism earlier than the central line or has a higher colony count, then it is not considered as CRBSI.

2. **Central line-associated bloodstream infection (CLABSI)** This is generally used in infection control surveillance. It is defined as a blood culture (a single blood culture for an organism not commonly present on the skin, and two or more blood cultures for an organism commonly present on the skin) in a patient who had central line at the time of infection or within 48 hours before the development of infection. This infection is not related to any other source of infection the patient may have had when she was admitted to the hospital.
3. The third type of infection associated with intravenous catheter is infection at the site of insertion of the catheter, which may be due to improper catheter care. They may be two types:
   i) **Exit site infection** Signs of inflammation are seen surrounding the catheter exit site confined to an area <2 cm in diameter and the presence of exudate which is culture-positive.
   ii) **Tunnel infection** In this case, inflammation extends along with the vein entry site beyond 2 cm, which is associated with pain and

tenderness along the subcutaneous track. The exudate from the exit site will show positive bacterial growth.

Health Care-Associated Pneumonia and Ventilator-Associated Pneumonia

Hospital-acquired pneumonia (HAP) is a lower respiratory infection that presents clinically 48 hours or more after hospitalisation and that was not incubating at the time of hospital admission.

Aspiration in unconscious patients and pulmonary ventilation or instrumentation may lead to nosocomial pneumonia, particularly in those with pre-existing cardiopulmonary disease. Multidrug-resistant *S. aureus* and gram-negative bacilli are the common pathogens.

Ventilator-associated pneumonia (VAP) is defined as pneumonia that occurs 48–72 hours after endotracheal intubation. There are new infiltrates with signs of systemic infection (fever, increased white blood cell count and detection of a causative agent from endotracheal or bronchoalveolar lavage). These findings are scored based on a clinical pulmonary infection score (CPIS). VAP is generally caused by multidrug-resistant strains of *Acinetobacter baumannii*, *Pseudomonas* and *Klebsiella* which have increased in the recent past.

Catheter-Associated Urinary Tract Infection (CAUTI)

This is one of the commonest health care-associated infections. Even with adequate precautions, catheterisation in hospitals leads to urinary infections in about 2 per cent of cases. The rate goes up to 50 per cent or more with indwelling catheters. *E. coli, Proteus, Pseudomonas aeruginosa* and other gram-negative bacilli and enterococcus are commonly associated with CAUTI. Mixed infection is common. Catheter-related infections can be prevented by strict asepsis during catheterisation. Indwelling catheters are to be used only when unavoidable, and care must be taken to ensure proper closing of drainage.

Health Care-Associated Wound Infections [Surgical Site Infections (SSI)]

Several factors influence the occurrence of postoperative wound infections, such as the site and duration of surgery, the health of the patient and skill of the operator. Most wound infections manifest within a week of surgery.
S. pyogenes and clostridial infections appear within a day or two, while staphylococcal infections typically take four or five days and gram-negative bacillary infections six or seven days to appear. Routine preoperative antibiotics do not prevent wound infections, though they may sometimes delay such infections.

Health Care-Associated Infections due to Hepatitis Viruses B and C (Transfusion-Associated Infections)

These infections are a serious risk for patients receiving blood transfusions or undergoing renal dialysis. Such infections are also a major risk for hospital personnel working with patients in dialysis and cardiac surgery units and in those who handle blood specimens. Although screening for hepatitis B surface antigen (HbsAg) and anti-hepatitis C virus antibody has markedly reduced the risk of acquiring such infections, other predisposing factors still exist. Strict attention to aseptic techniques would greatly reduce this risk for hospital personnel.

Health Care-Associated Episodes of Acute Gastroenteritis

Outbreaks of *Salmonella* food poisoning, enterotoxic manifestations due to staphylococcal contamination of cooked food, and outbreaks of diarrhea due to *Escherichia coli* and certain enteroviruses are well-known examples of health care-associated gastroenteritis.

Antibiotic-associated diarrhea occurs due to the prolonged use of antibiotics like fluoroquinolones, cephalosporins, penicillins (ampicillin, amoxicillin) and clindamycin. Diarrhea occurs within a few days of starting the antibiotic. The normal gut flora gets suppressed due to the antibiotics. This leads to the overgrowth of *C. difficile* which secretes the exotoxins: (i) toxin A (TcdA) and (ii) toxin B (TcdB), which are both glucosylating toxins and (iii) transferase, a binary toxin. These toxins act on the colonic epithelium and immune cells and induce events that result in fluid secretion,

inflammation and tissue damage, leading to diarrhea.

Health Care-Associated Episodes of Tetanus

Factors that lead to such episodes include failure to implement strict aseptic precautions during surgical procedures, the use of contaminated or improperly sterilised dressings and suture material, improper (or no) disinfection of the site of intramuscular injections, particularly those given in the gluteal region, and inadequate care while cutting the umbilical cord of the newborn child. Such episodes in the newborn are no longer recorded due to stringent vaccination programme for antenatal women.

Other Infections

Needlestick and sharps injuries Every percutaneous needlestick and sharps injury carries a risk of infection by bloodborne pathogens like **hepatitis B, C and D and HIV besides other infections.** These infections can arise due to:

- Devices with hollow bore needles, suture needle, IV stylet
- Needle devices taken apart or manipulated by the health care worker while drawing blood (phlebotomy) and devices that need to be detached after use
- Syringes that retain an exposed needle after use
- Butterfly needles that are attached to tubing and cannot be disposed in a sharps container

In the event of a needlestick or sharps injury in a health care setting, a protocol needs to be followed immediately to prevent the transmission of infection. The event needs to be reported to the infection control nurse and medical officer. As a part of the injury reporting system, the source patient is tested for HIV, hepatitis B and hepatitis C after seeking consent.

- If the person is found to be positive for HIV or if the status is unknown, post-exposure prophylaxis (PEP) is initiated (as directed by the CDC and WHO). The course is a four-week

regimen of two drugs—zidovudine (ZDV) and lamivudine (3TC). If the person tests negative, no PEP is required.
- If the patient tests positive for hepatitis B, the health care worker needs to be vaccinated with HBIG and HB vaccines; if the worker is already vaccinated, no treatment is required.
- No treatment is currently recommended for a health care worker who has been exposed to hepatitis C.

Prevention of needlestick injury is by following safe practices: ensuring that re-capping is not done, that needles and sharps are disposed in puncture-proof containers and that sharps containers are emptied before they are full.

SOURCES AND RESERVOIRS OF HEALTH CARE-ASSOCIATED INFECTIONS

Endogenous Source of Infection

This is often the most common source. Here, the infecting microorganism is not pathogenic under normal conditions but when there is an underlying (usually debilitating) disease or when invasive diagnostic and therapeutic procedures (including the use of immunosuppressives and antibiotics) have been performed, this microbe can reproduce, spread and localise at a site where it may produce infection.

Cross-Infection

In this condition, the microorganism spreads by:

- Direct contact between an infected patient and another individual (by droplets of saliva or respiratory secretions from the patient's hands)
- Air (dust from a fabric that carries the patient's normal microbial flora)
- Hospital personnel, on whose hands or clothes the microorganisms settle, from where the microbes may be directly transmitted to a patient; alternatively, the organisms may be

harboured in the respiratory or intestinal mucous membranes of the hospital personnel, where multiplication occurs, followed by transmission to patients by direct contact or air

Infections From Environmental Sources

In these types of infections, the microorganisms concerned spread from:

- Hospital air, which usually harbours many bacteria that are often pathogenic and resistant to multiple drugs.
- Surfaces, which are contaminated by the patient's secretions, excreta, blood or body fluids, or by animals and insects.
- Inanimate objects, which are contaminated by patients, the hands of healthy or unhealthy hospital personnel, visitors, food or infected water, animals and insects. Examples of objects contaminated by patients include hospital equipment (sanitary installations, lights, tables, beds) and medical equipment (endoscopes, catheters, vesical probes, needles, lancets, spatulas and instruments used for invasive and non-invasive procedures). Examples of objects contaminated by hospital staff include items in the kitchen, laundry and treatment room.

Modes of Transmission of Microorganisms

In health care settings, especially in hospitals, the four main routes of transmission of microorganisms are:

i) Aerosols
ii) Oral route
iii) Direct contact
iv) Parenteral route

MEASURES TO CONTROL INFECTION IN THE HEALTH CARE SETTING

Every health care institution should have a properly constituted and functioning infection control team. The basic principles of personal and institutional hygiene should be strictly observed to ensure patient safety.

The aims of infection control are as follows:

1. Reduce the microbial population of the hospital environment
2. Eliminate the danger of transmission of microorganisms from one individual to another:

    - From hospital personnel to patient
    - From patient to personnel to patient
    - From patient to patient

3. Manage linen, equipment and other inanimate objects to prevent them from becoming sources of cross-contamination
4. Practice safe methods of biomedical waste disposal (Chapter 65)

Observing standard precautions is crucial to the success of infection control measures in a health care setting.

Standard Precautions

Standard precautions are the minimum infection prevention practices that apply to all patient care, regardless of the suspected or confirmed infection status of the patient, in any setting where health care is delivered (Fig. 62.1). They include: hand hygiene, use of personal protective equipment, safe injection practices, safe handling of potentially contaminated equipment or surfaces in the patient environment and respiratory hygiene/cough etiquette (*Source: www.cdc.gov/HAI/settings/outpatient/outpatient-care-gl-standared-precautions.html, accessed 8 February 2013*).

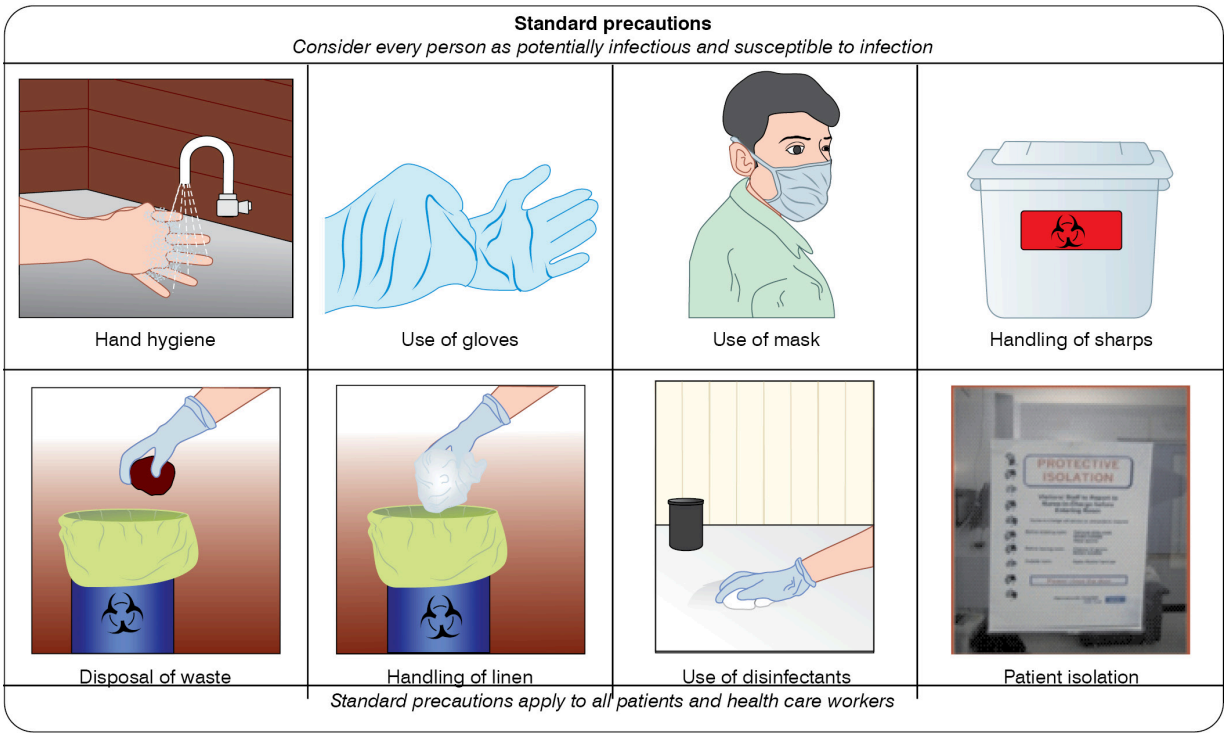


Fig. 62.1 Standard precautions (*Source:* Department of Clinical Microbiology, Christian Medical College and Hospital, Vellore)

1. Hand hygiene

Good hand hygiene, including the use of alcohol-based hand rubs and handwashing with soap and water (Fig. 62.2), is critical to reducing the risk of spreading infections in ambulatory care settings.

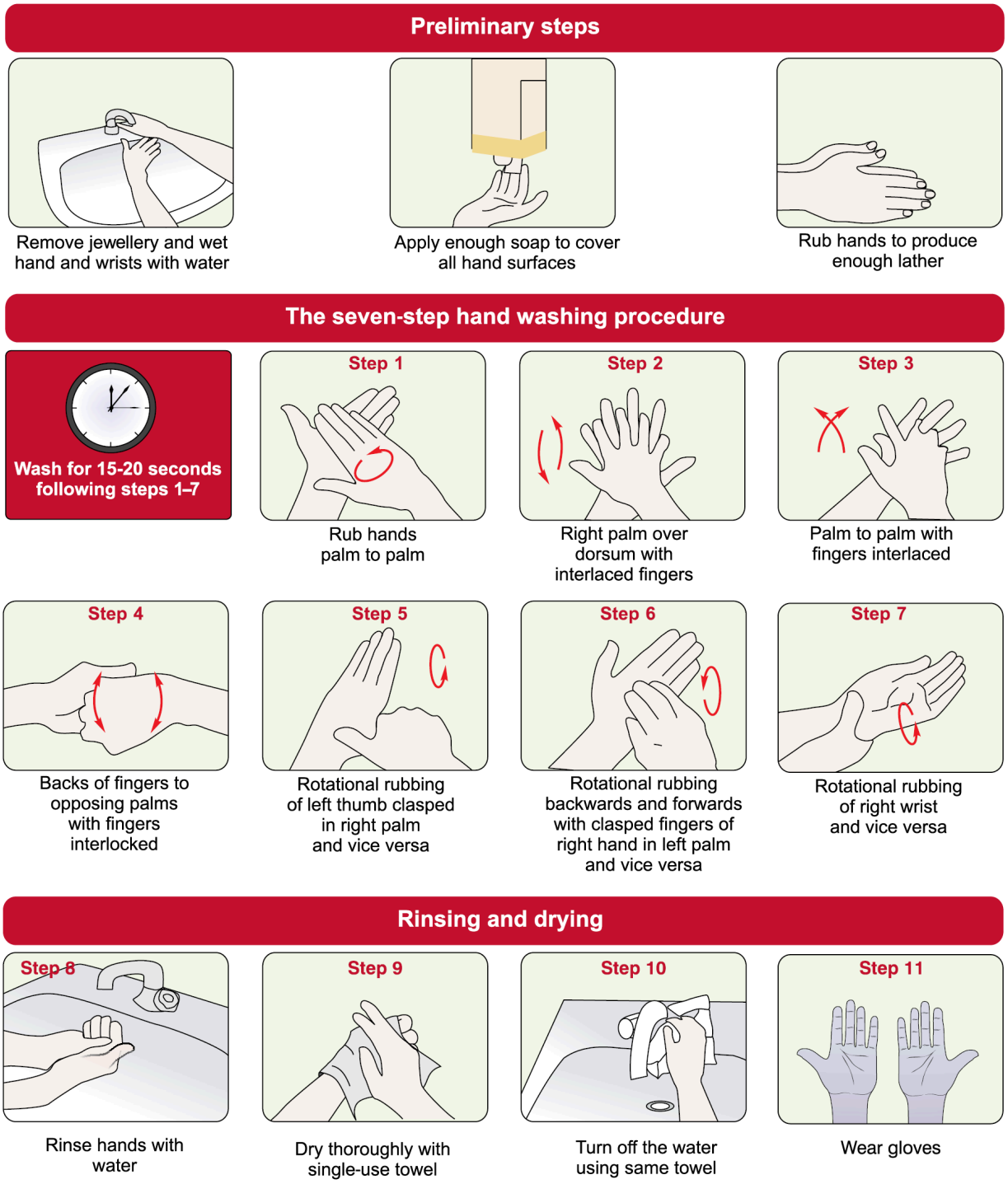

Preliminary steps
Remove jewellery and wet hand and wrists with water
Apply enough soap to cover all hand surfaces
Rub hands to produce enough lather
The seven-step hand washing procedure
Wash for 15-20 seconds following steps 1–7
Step 1
Rub hands palm to palm
Step 2
Right palm over dorsum with interlaced fingers
Step 3
Palm to palm with fingers interlaced
Step 4
Backs of fingers to opposing palms with fingers interlocked
Step 5
Rotational rubbing of left thumb clasped in right palm and vice versa
Step 6
Rotational rubbing backwards and forwards with clasped fingers of right hand in left palm and vice versa
Step 7
Rotational rubbing of right wrist and vice versa
Rinsing and drying
Step 8
Rinse hands with water
Step 9
Dry thoroughly with single-use towel
Step 10
Turn off the water using same towel
Step 11
Wear gloves

Fig. 62.2 Eleven steps to hand washing and drying

Key situations where hand hygiene should be observed include (Fig. 62.3) the following:

i) Before touching a patient, even if gloves are to be worn

ii) Before coming out of the patient's care area after touching the patient or the patient's immediate environment
iii) After contact with blood, body fluids or excretions or wound dressings
iv) Prior to performing any aseptic task (e.g., placing an intravenous line or preparing an injection)
v) If hands are likely to move from a contaminated body site to a clean body site during patient care and after removal of gloves

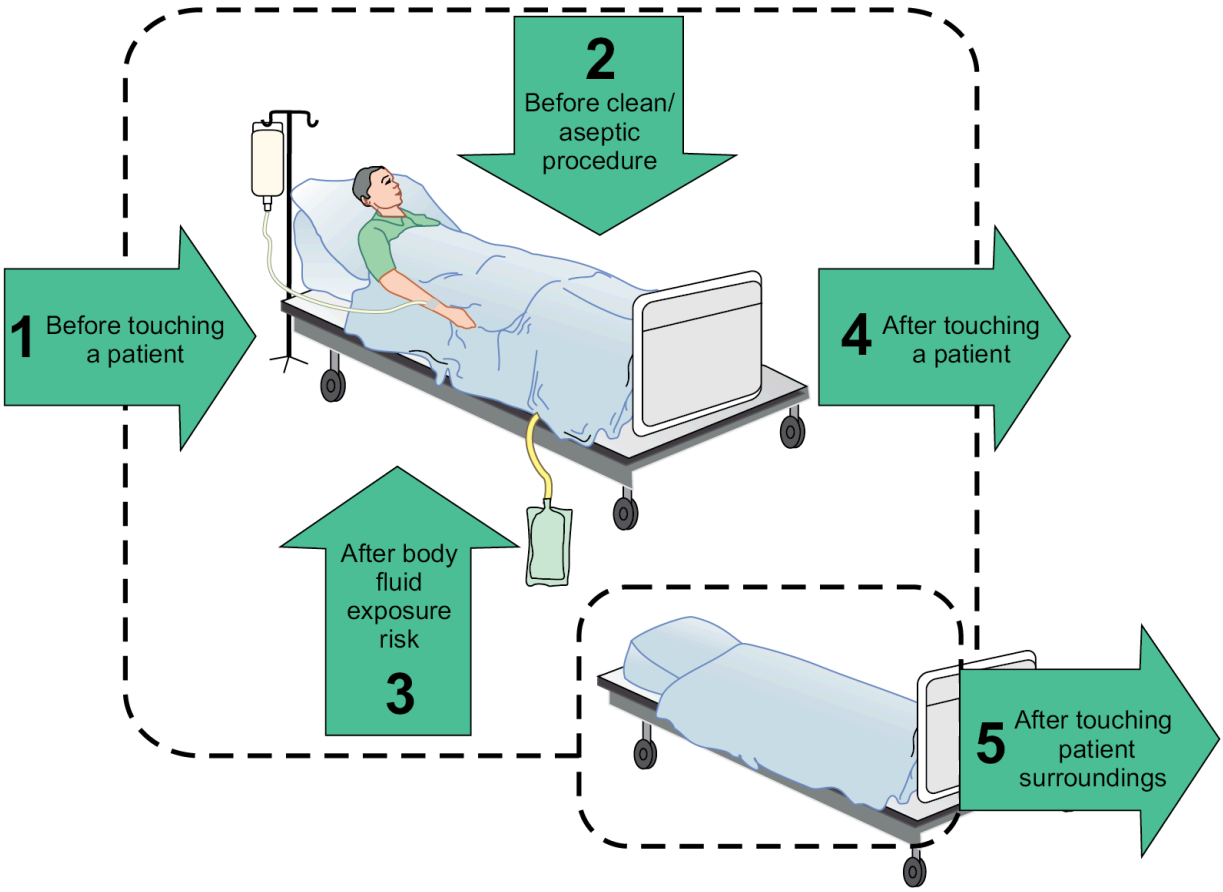


Fig. 62.3 Five moments for hand hygiene

2. Personal protective equipment (PPE)

This refers to wearable equipment intended to protect health care workers (HCWs) from exposure to or contact with infectious agents; examples include gloves, gowns, face masks, respirators, goggles and face shields.

The following are recommended for the use of gloves:

- Gloves should be worn when there is a possibility of contact with blood, body fluids, mucous membranes, non-intact skin or contaminated equipment.
- The same pair of gloves should not be worn for the care of more than one patient.
- Gloves should not be washed for the purpose of reuse.
- The steps for hand hygiene should be performed immediately after removing the gloves.

Other key recommendations are as follows:

- A gown should be worn to protect skin and clothing during procedures or activities where contact with blood or body fluids is anticipated.
- The same gown should not be worn for the care of more than one patient.
- Mouth, nose and eye protection should be in place during procedures likely to generate splashes or sprays of blood or other body fluids.
- A surgical mask should be worn when placing a catheter or injecting material into the spinal canal or subdural space.

3. Injection safety (safe injection practices)

This refers to practices intended to prevent the transmission of infectious diseases between patients or between a patient and a health care worker (HCW) during the preparation and administration of parenteral medications.

The following are recommended:

- Aseptic techniques should be used when preparing and administering medications.
- Access diaphragms of medication vials should be cleaned with 70% alcohol before inserting a device into the vial.
- Medications should never be administered from the same syringe to multiple patients, even if the needle is changed or the injection

is administered through an intervening length of intravenous tubing.

- A syringe should not be reused to enter a medication vial or solution.
- Medications should not be administered to more than one patient from single-dose or single-use vials, ampoules or bags or bottles of intravenous
- Fluid infusion or administration sets (e.g., intravenous tubing) should not be used for more than one patient.
- Multi-dose vials should be dedicated to a single patient whenever possible.
- Used needles should not be capped on any account.

4. Environmental cleaning

Cleaning refers to the removal of visible soil and organic contamination from a device or environmental surface using the physical action of scrubbing with a surfactant or detergent and water, or an energy-based process (e.g., ultrasonic cleaners) with appropriate chemical agents. This process removes large numbers of microorganisms from surfaces and must always be performed before disinfection.

Disinfection is generally a less lethal process of microbial inactivation (compared to sterilisation) which eliminates virtually all recognised pathogenic microorganisms but not necessarily all microbial forms (e.g., bacterial spores).

5. Medical equipment

Medical equipment may be **reusable** or **single-use**. Reusable medical equipment (e.g., endoscopes) should be accompanied by instructions for cleaning and disinfection or sterilisation as appropriate. Single-use devices are labelled by the manufacturer for only a one-time use and do not have reprocessing instructions.

Health care facilities should ensure that reusable medical equipments (e.g., blood glucose meters and other point-of-care devices, surgical instruments and endoscopes) are cleaned and reprocessed appropriately before being used on another patient.

6. Respiratory hygiene/cough etiquette

This represents an element of standard precautions that highlights the need for prompt implementation of infection prevention measures at the first point of encounter with the facility/ambulatory settings. Any individual with signs of illness including cough, congestion, rhinorrhea or increased production of respiratory secretions needs to be promptly identified when entering the facility and should be monitored throughout the duration of the visit.

To prevent transmission of respiratory secretions in individuals (patients and accompanying individuals) who have signs and symptoms of a respiratory infection, notices should be posted at entrances with instructions to all individuals to cover their mouths/noses when coughing or sneezing, to use and dispose of tissues and to perform hand hygiene after hands have been in contact with respiratory secretions (Fig. 62.4).

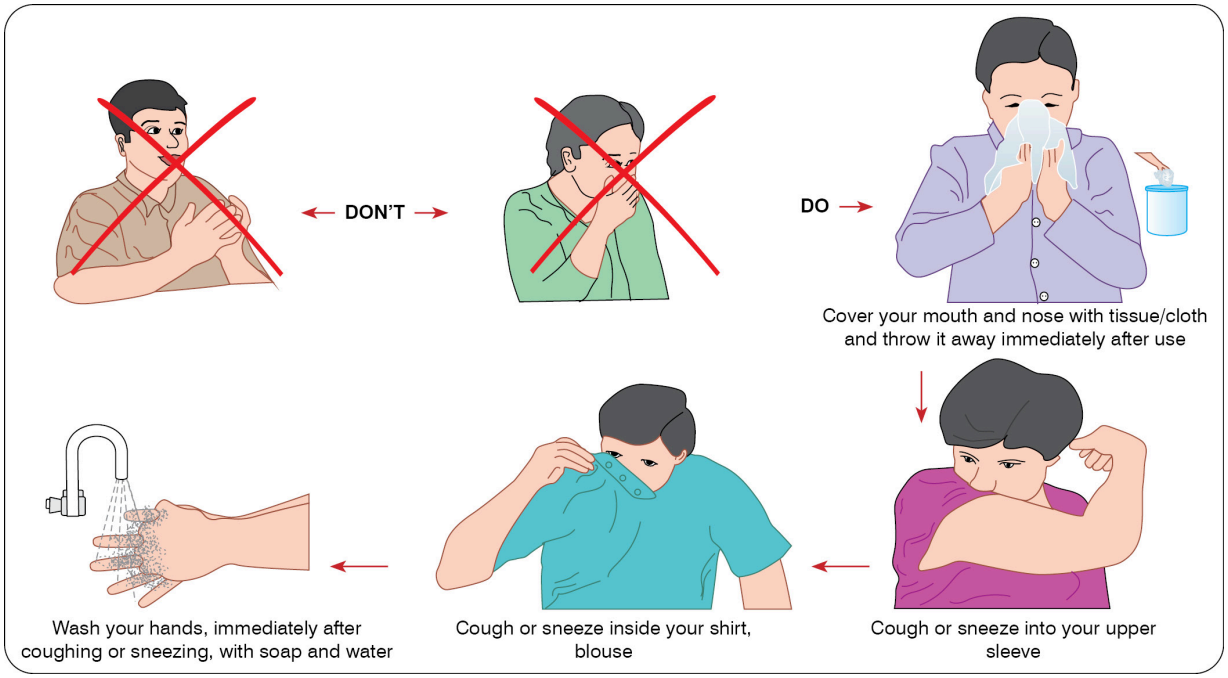

DON'T
DO
Cover your mouth and nose with tissue/cloth and throw it away immediately after use
Cough or sneeze into your upper sleeve
Cough or sneeze inside your shirt, blouse
Wash your hands, immediately after coughing or sneezing, with soap and water

Fig. 62.4 Respiratory hygiene while coughing and sneezing

7. Isolation

All patients admitted with contagious infections must be isolated. Patients with MRSA and multidrug-resistant organisms (MDRO), which are resistant to three or more classes of antibiotics, need to be isolated and

treated by barrier nursing. The nursing care is individualised so that the infection does not spread to other patients via the nurse. All personal protective equipment is dedicated to single care.

Precautions in the Operating Theatre

Conventional or **plenum** type ventilation in operating theatres maintains approximately 20 air changes per hour; however, in laminar flow operating theatres, there may be as many as 300 air changes per hour. Airborne organisms typically occur in counts of between 150 and 300 cfu/m^3 (cfu = colony forming units) in conventional operating theatres, while with laminar flow ventilation, a count of 10 cfu/m^3 or less is achieved.

It is recommended that for conventional operating theatres, the bioload (bacteria-carrying particles per cubic metre) should not exceed 35 in an empty theatre or 180 during an operation. For ultraclean air operating theatres, the bioload should be less than 1.0 in the centre of an empty theatre and less than 10 during an operation, and should not exceed 20 at the periphery.

INVESTIGATION AND FOLLOW-UP OF OUTBREAKS OF DISEASE

Even with all preventive measures in place, outbreaks of health care-associated infections may still occur. In the unfortunate event of an outbreak in any area of the hospital, investigations are needed to determine whether:

- carriers are responsible for the outbreak by providing a continuous reservoir of pathogenic microorganisms.
- there has been a single source of pathogens (e.g., food served at a particular meal).
- there have been deficiencies in the technique adopted in the day-to-day operations in the affected area.

In the case of an outbreak, the infection control team decides on preventive measures as soon as the outbreak is detected. Environmental surveillance and screening of health care personnel is done to detect the

source and necessary containment is done by treating (decolonising) the source individual and relocating him to a non-patient-care area. Adequate decontamination and disinfection of the surrounding area is done to remove the microbial source.

If the outbreak is caused by a notifiable disease, it should be reported to the authorities.

MONITORING AND REGULATION OF HCAI: HOSPITAL INFECTION CONTROL COMMITTEE (HICC)

With increasing incidence of HCAI, most hospitals have to monitor, regulate, implement and take preventive measures to control and contain HCAI. A hospital infection control committee (HICC) is constituted to plan, monitor, evaluate, update, and educate health care professionals on standard infection control practices. The responsibility of the committee is to:

- Meet periodically to evaluate the infection rates in the hospital
- Monitor infection control practices
- Look at infrastructural corrections required to contain spread of infection
- Detect hospital outbreaks of infections

The committee is chaired by the medical superintendent of the hospital, while the head of the microbiology department is the member secretary who coordinates the meetings and HICC activities. The other important member is the infection control nurse (ICN) who visits all areas of patient care, OTs, Central Sterile Supply Department (CSSD), hospital laundry and biomedical waste units to monitor the practices. The other members include the heads of surgical and medical specialties, engineering and nursing, heads of housekeeping and hospital kitchen to prevent food-borne infection outbreaks.

Key points

- A health care-associated infection (hospital-acquired infection, nosocomial infection) is one that develops in a hospitalised patient but was not present or was not in incubation at the

time of admission; such an infection usually becomes evident 48–72 hours after admission to the hospital and sometimes manifests only after discharge.

- Hospital infections are usually exogenous, the source being any part of the hospital ecosystem (people, objects, food, water or air).
- Health care-associated infections manifest as wound infections, urinary tract infections, respiratory infections (nosocomial pneumonia), bacteremia and septicemia.
- Health care-associated infections may occur sporadically or as outbreaks; possible sources of infection are investigated by routine bacteriological methods to identify the source.
- Standard precautions are the minimum infection prevention practices that apply to all patient care, and include hand hygiene, use of personal protective equipment, safe injection practices, safe handling of potentially contaminated equipment or surfaces in the patient environment and respiratory hygiene/cough etiquette.
- For conventional operating theatres, the bioload (bacteria-carrying particles per cubic metre) should not exceed 35 in an empty theatre or 180 during an operation; for ultraclean air-operating theatres, the bioload should be less than 1.0 in the centre of an empty theatre and less than 10 during an operation, and should not exceed 20 at the periphery.

Questions

Short Answers

1. Types of health care-associated infections
2. Hand hygiene
3. Personal protective equipment
4. Safe injection practices
5. Role of HICC in infection control in the hospital

Part VI: Clinical Microbiology as Applied to Infectious Diseases

Chapter 63

Immunoprophylaxis

Chapter navigation

- Active immunisation
- Passive immunisation

INTRODUCTION

Immunisation has been an important contribution of microbiology to medicine. It is one of the most effective methods of controlling infectious diseases. By systematic active immunisation programmes, many developed countries have virtually eliminated **vaccine-preventable diseases (VPD)** such as diphtheria, pertussis, tetanus, measles, mumps, rubella and poliomyelitis. The global eradication of smallpox has, of course, been the crowning glory of immunisation.

Immunoprophylaxis may be in the form of **active or passive immunisation** of individuals or selected groups exposed to the risk of specific infections.

ACTIVE IMMUNISATION

Active immunisation is carried out using specific vaccines that provide protection against specific diseases. Vaccination evokes both humoral and cell-mediated immunity. Active immunity is associated with specificity and immunological memory (primary and secondary responses). The vaccines used for immunisation may be live, killed or in other forms like toxoids, subunits and recombinant vaccines
(Table 63.1).

Table 63.1 Types of vaccines

Vaccine type	Examples
Live attenuated	Measles, mumps, rubella, varicella-zoster, BCG, rotavirus, oral polio (Sabin)
Killed vaccine	Rabies, pertussis, cholera, hepatitis A, killed polio (Salk)
Recombinant subunit	Hepatitis B
Toxoid	Tetanus, diphtheria
Protein conjugated	*H. influenzae* type-b (Hib), meningococcal, pneumococcal (13PCV)
Polysaccharide	Meningococcal and pneumococcal vaccines

Live Attenuated Vaccines

Organisms that have been treated so that they lose their ability to cause disease (pathogenicity) while retaining their ability to induce an immune response (immunogenicity) and generate memory cells are said to be **attenuated**. They are grown in altered culture conditions for prolonged periods to achieve attenuation. Live vaccines are more potent immunising agents than killed vaccines as they multiply, resulting in higher antigenic dose than what is injected, thus evoking an immune response. Live vaccines retain all major and minor antigenic components. Oral polio, measles, mumps and BCG vaccines are common examples. **Live attenuated vaccines are avoided in immunocompromised individuals due to the danger of the vaccine strain causing the disease in the absence of host immune response.**

Live vaccines

- Primary course provides relatively long-lasting protection.
- The immune response is broad, providing both humoral (antibody-mediated) and cell-mediated immunity.
- They exhibit wide antigenic diversity.
- Live vaccines have demonstrated good cross-reactivity against heterologous strains.

Killed or Inactivated Vaccines

Killed vaccines are relatively less efficacious as the pathogens are inactivated by heat or chemicals, and hence do not cause disease. Killed

vaccines require primary and booster doses. They are stable and safer than live vaccines and do not require cold facilities for storage; however, they generate a weaker immune response. Examples of killed vaccines are the cholera, influenza, pertussis, plague, rabies and hepatitis A vaccines.

Killed vaccines

- They are safer than live vaccines.
- They do not result in infection and, therefore, the immune response produced is not as complete as that which is stimulated by a live vaccine.
- Repeat doses have to be given.

Toxoids

Exotoxins produced by certain microorganisms are responsible for causing diseases such as diphtheria and tetanus. Toxoids used as vaccines are purified toxins inactivated by treatment with formaldehyde. Antibodies generated against toxoids neutralise the toxic moiety produced during infection, rather than acting upon the pathogen. Some toxoids are mixed with other vaccines to enhance their antigenicity. In the DPT (diphtheria, pertussis, tetanus) vaccine, diphtheria and tetanus toxoids are combined with the pertussis vaccine (which acts as an adjuvant). The two toxoid vaccines commonly used for immunisation are diphtheria and tetanus.

Cellular Fractions (Bacterial Polysaccharides)

Certain vaccines are prepared from extracted cellular fractions of microorganisms. Examples are the meningococcal vaccine which is prepared from the polysaccharide antigen of the cell wall and the pneumococcal vaccine from the polysaccharides contained in the capsule of the organism. Their safety and efficacy are high but they are of limited use.

Conjugate Vaccines

Vaccines with carbohydrate polysaccharide antigens require a protein conjugate to increase the immunogenicity, e.g., heptavalent pneumococcal conjugate vaccine (7PCV).

Other Vaccines

Subunit vaccines

They are produced from purified macromolecules derived from immunogenic components of pathogenic microorganisms by recombinant DNA technology. Other candidate vaccines for influenza, HBsAg and HIV are now being incorporated in micelles (lipid molecules that cluster together in aqueous solutions and trap the antigen), liposomes, isocoms (antigens trapped in dendritic cells) and virosomes (these are derived from the virus and are used as vehicles for antigen presentation to the immune system) for better delivery.

Recombinant vaccines

Genes encoding antigens are cloned in bacteria, yeast and some mammals using recombinant technology. The expressed antigenic proteins are purified and used as vaccines. Examples are the hepatitis B and pertussis vaccines. Surface antigen of the hepatitis virus was the first recombinant vaccine to be cloned in yeast. Since purified proteins are used, adverse reactions are minimal.

DNA vaccines

In contrast to whole bacterial or viral vaccines, DNA vaccines are made of small, circular pieces of the DNA (plasmid) of the microorganism that has been genetically engineered to produce one or more specific proteins of the pathogen against which immunity is desired. The gene is incorporated into a bacterial plasmid by recombinant technology. The bacteria replicate to produce large quantities of the plasmid, which are then purified. This forms the source of the required DNA which is delivered by intramuscular injection, dermal patches or electroporation (EP) (EP refers to the application of short electrical pulses to the delivery tissue) for DNA uptake by the cells. These DNA vaccines can activate $CD8^+$ cytotoxic T cells (CTL) to evoke an immune response. Vaccines that are under trial are HIV, influenza, hepatitis B and C, HPV and malaria.

Edible vaccines

Genes that code for the particular antigen are introduced into plants by a process of transformation. These plants produce the encoded proteins (which are antigenic proteins and act like the subunit vaccines) which can

stimulate an immune response in the human body. The altered plants are known as **transgenic plants**. The edible vaccines do not have the pathogenic genes. The vaccine is given orally and acts through the intestinal mucosa. Edible vaccines have been tried for cholera, measles and hepatitis C and E. Banana, tomato, potato, lettuce and rice act as the transgenic plants.

Routine Immunisation Schedules

Routine immunisation schedules have been developed for different countries and are modified from time to time, based on the prevalence of infectious diseases, their public health importance, availability of suitable vaccines, their cost–benefit factors and logistics. In India, the Expanded Programme on Immunisation (EPI) and the Universal Immunisation Programme (UIP) have been able to provide protection against VPDs for much of the target population.

WHO Universal Immunization Programme

The Expanded Programme of Immunization (EPI) has now been named as the **Universal Immunization Programme (UIP) in India.**

In India, EPI and UIP have led to a significant decline in the recorded incidence of vaccine-preventable diseases, as well as in infant and child mortality.

The following vaccine-preventable diseases are covered under the Universal Immunization Programme:

1. **Diphtheria**
2. **Pertussis**
3. **Tetanus**
4. **Polio**
5. **Measles**
6. **Hepatitis B**
7. **Tuberculosis**
8. **Pneumococcal disease**
9. *Haemophilus influenzae* **type B related illnesses**
10. **Diarrhea caused by rotavirus**
11. **Rubella**

National Immunisation Schedule

The National Immunisation Schedule in force in India is presented in Table 63.2.

India has been polio-free along with the entire South-East Asia Region since 2014, as certified by WHO. In January 2018, India marked seven years since the last case of polio was reported in the country. But until the disease is eradicated, children are vaccinated across the country to maintain high levels of childhood immunity.

Table 63.2 Vaccination as per the National Immunisation Schedule by the Government of India

Age	Vaccine
Birth	BCG, OPV(0), Hep B birth dose (to be given at the place of delivery)
6 weeks	OPV1, Penta1 (DPT+HepB+HiB)
10 weeks	OPV2, Penta2 (DPT+HepB+HiB)
14 weeks	OPV3, Penta3 (DPT+HepB+HiB)
9 months	IPV, MMR-2, MR/Measles, JE vaccine-1 where applicable
16–24 months	MMR-1, OPV booster, DPT 1st booster
5–6 years	DPT 2nd booster, JE vaccine-2
10 years	TT-1
16 years	TT-2

New vaccines for children
Newer vaccines in the schedule like **HiB, rotavirus vaccine (Rotarix), pneumococcal vaccine** and **7PCV or 13PCV** are available for the immunisation of children.

PASSIVE IMMUNISATION

In some diseases, the need for immune intervention is so acute that treatment cannot await the recipient mounting his own immune response. Also, sometimes the recipient may be incapable of generating an immune response. In such cases, pre-formed antibodies are transferred to the patient and human or animal sera used for passive immunisation.

Human Sera

Normal immunoglobulins can be injected in two forms: pooled and specific.

i. **Pooled immunoglobulins** are used for short-term prophylaxis in cases of exposure to hepatitis A or measles.
ii. **Hyperimmune immunoglobulins** are prepared from the plasma of patients in the convalescent phase of that infection. Passive immunisation is deployed using specific human immunoglobulins (IG) for protection against different diseases. Examples are hepatitis B (HBIG), tetanus (HTIG), rabies (HRIG), vaccinia (HVIG), etc.

Administration of human sera Human sera are injected intramuscularly. For rabies, half the dose is given around the bite wound and the other half, intramuscularly.

Animal Sera

Equine sera were used earlier but now human hyperimmune serum is preferred to animal sera to avoid hypersensitivity.

COMBINED ACTIVE AND PASSIVE IMMUNISATION

A combination of active and passive immunisation is preferred when immediate passive immunity has to be provided until active immunisation generates specific immune responses to the particular disease/infection. Here, a combination of vaccines (diphtheria/tetanus and rabies) is given simultaneously but injected at two different sites.

INDIVIDUAL IMMUNISATION

The vaccines offered under national programmes are limited by economic considerations and so certain important vaccines may be omitted because they are costly. These may be supplemented by individual initiative, whenever possible.

Hepatitis B Vaccine

Many developing countries, including India, have high endemicity for this virus. Perinatal transmission and acquisition of the viral infection in the first five years of life are common in such areas. Inclusion of the hepatitis B vaccine in routine childhood immunisation will, therefore, be beneficial. The fact that a quarter to half the adult dose of the vaccine is adequate for

children brings down its cost. The recent reduction in the cost of the vaccine as a result of indigenous manufacture has made mass vaccination more feasible in some of the states in India. Several health care guidelines advocate HepB vaccine to all health care professionals.

Varicella Vaccine

Chickenpox , which is a fairly mild disease in children, can be very serious and even fatal for adults. The varicella vaccine had been used for many years in immunocompromised children. Recently, with the development of a more stable and effective vaccine, its scope has been extended for general use for the prevention of varicella and herpes zoster. All children should routinely receive the first dose of varicella vaccine (live-attenuated Oka strain of VZV) at 12 to 15 months of age. The second dose of the vaccine is recommended at 4 to 6 years of age. Vaccination is contraindicated in pregnancy.

Typhoid Vaccine

Typhoid fever continues to be a major public health problem in the developing countries. Two recent typhoid vaccines, the live oral Gal-E mutant vaccine and the injectable purified Vi polysaccharide vaccine, may be beneficial because they offer prolonged protection and are free from reactions. They are recommended for immunisation of children aged five years or above and so may be employed at school entry.

Immunoprophylaxis of individual diseases has been described in the respective chapters.

VACCINATION DURING OUTBREAKS

Population protection during outbreaks or epidemics of infections is an important measure of infection containment. Some of the vaccines used are flu vaccines (seasonal flu, H1N1 vaccines), cholera vaccines, Japanese encephalitis vaccine, etc.

NEWER VACCINES IN THE EXPERIMENTAL OR CLINICAL TRIAL STAGES

Several vaccines are in the developmental stages or under clinical trial.

Some of the notable ones are for the following diseases:

- Dengue
- Chikungunya
- HIV
- Malaria
- Group B streptococci
- Pneumococci
- Nipah
- Zika
- Influenza (Universal Influenza Vaccine)
- COVID-19

Immunomodulation

In certain instances, the immune system needs to be modulated or suppressed. Immunomodulators are agents that weaken immunocompetent cells which, in turn decreases the inflammatory response. Immunomodulators are most often used in organ transplantation to prevent the rejection of the new organ and in autoimmune disorders such as rheumatoid arthritis. Other conditions that have been associated are Crohn's disease, irritable bowel syndrome and ulcerative colitis, where the immune system is overactive. The drugs often used are cycloserine, azathioprine and 6-mercaptopurine. Corticosteroids are more commonly used as mild immunomodulators.

Cold chain

The purpose of the vaccine 'cold chain' is to maintain product quality from the time of manufacture until the point of administration by ensuring that vaccines are stored and transported within WHO-recommended temperature ranges. Vaccine vial monitors attached to the vaccine container are used for temperature monitoring. These are chemical indicator strips which change colour in a gradient manner if the temperature goes beyond the prescribed limit (Fig. 63.1a and b).

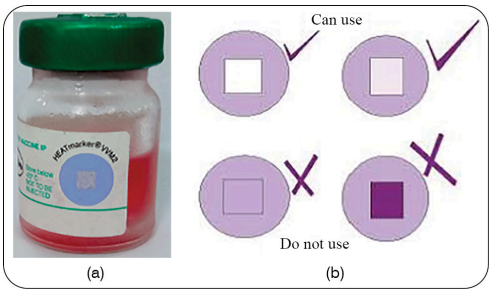


Fig. 63.1 (a) Vaccine vial monitor and (b) interpretation of the colour change (*Source:* (a) Pharmacy, Pondicherry Institute of Medical Sciences, Puducherry)

Key points

- Immunoprophylaxis provides specific protection against infectious diseases by stimulating the immune system.
- Active immunisation involves both humoral and cell-mediated immunity and is associated with specificity and immunological memory.
- Vaccines used for immunisation may be live, killed or in other forms like toxoids, subunits and recombinant vaccines.
- The transfer of pre-formed antibodies, human sera or animal sera is used for passive immunisation.
- Immunoprophylaxis may be in the form of routine immunisation, which forms part of basic health care or immunisation of individuals exposed to the risk of specific infections.
- In the National Immunisation Schedule in India, BCG, oral polio, DPT and measles vaccines are given to infants and children at different intervals according to the schedule.
- Newer vaccines like HiB, rotavirus vaccine and pneumococcal conjugate vaccine are available for use.

Questions

Essays

1. Define the term passive immunisation. Explain how human sera/immunoglobulins are beneficial in this.
2. Describe comprehensively the National Immunisation Schedule in force in India.

Short Answers

1. Live attenuated vaccines
2. Killed vaccines
3. Toxoids
4. Subunit vaccines
5. Passive immunisation
6. National Immunisation Schedule

Part VI: Clinical Microbiology as Applied to Infectious Diseases

Chapter 64

Bacteriology of Water, Air, Milk and Food

Chapter navigation

- Bacteriological examination of water
  - Microbial (biological) quality
  - Chemical quality
  - Physical quality
- Air quality testing
- Bacterial examination of milk
- Sterilisation of milk

BACTERIOLOGY OF WATER

Drinking water is the most essential component of life. For this reason, it must be acceptably safe, that is, free from physical, chemical and microbiological contamination. According to the World Health Organization's 2017 report, safe drinking water is water that "does not represent any significant risk to health over a lifetime of consumption, including different sensitivities that may occur between life stages." Another concept is of '**wholesome' water,** which is water that is fit to use for drinking, cooking, food preparation or washing without any potential danger to human health by meeting the requirements of regulations made under Section 67 (Standards of Wholesomeness) of the *Water Act 1991*. These stipulate the criteria that the water must meet in order to protect the health of the population. These parameters include limits on:

- Microbiological (biological) quality (including levels of bacteria and oocysts)
- Chemical quality (including levels of metals, solvents, pesticides and hydrocarbons)
- Physical qualities (including colour, taste and odour)

Many major human diseases, for example, typhoid fever, cholera and other diarrheal diseases, poliomyelitis and viral hepatitis A and E are water borne. These pathogens reach water sources through fecal or sewage pollution. It is essential to treat water suitably to remove and prevent such contamination. To ensure the safety of such protected water supplies, regular bacteriological surveillance is recommended.

Natural water sources, even when unpolluted, frequently contain bacteria, such as species of *Pseudomonas, Serratia, Flavobacterium, Chromobacterium, Acinetobacter* and *Alcaligenes*. Soil bacteria, such as aerobic spore-forming bacilli, and those found on decaying vegetation, such as the *Enterobacter* species, may also be washed into natural waters during the rains; fortunately, these are harmless. Only pathogens introduced into water polluted by excreta or sewage pose a risk to human health (Table 64.1).

Table 64.1 Pathogens that spread through water

In the community		In the hospital (Rangel-Frausto, 2002)		
Organism	**Disease caused**	**Organism**	**Found in**	**Disease caused**
A. Bacteria 1) *Shigella* spp. 2) *Vibrio cholerae* 3) *Salmonella enterica* serotypes typhi and paratyphi A, B, C 1) *Leptospira* spp.	A. **Bacteria** 1) Bacillary dysentery 2) Cholera 3) Enteric fever 4) Leptospiremia, leptospiruria	**Bacteria** 1) *Legionella pneumophila*	1) Hospital water, cooling towers	1) Pneumonia
		2) *Serratia marcescens*	2) Water of humidifiers	2) Pneumonia
		3) *Mycobacterium xenopi*	3) Hot water taps	3) Pneumonia
		4) *Pseudomonas paucimobilis*	4) Water bottles for tracheal suction	4) Pneumonia
B. Parasites 1) Protozoa a) *Giardia lamblia* b) *Entamoeba histolytica* 2) Helminths a) *Schistosoma haematobium* b) *Dracunculus medinensis*	**B. Parasites** 1) Protozoa a) Diarrhea, malabsorption b) Amoebic dysentery 2) Helminths a) Hematuria b) Skin lesions	5) *Mycobacterium chelonae*	5) Contaminated equipment and water tanks	5) Otitis and nasal septum cellulitis
		6) *Acinetobacter* spp.	6) Water bath used to thaw fresh plasma	6) Bacteremia
		7) *Pseudomonas aeruginosa*	7) Water bath used to thaw cryoprecipitate; tub water	7) Bacteremia; folliculitis and skin infections
C. Due to viruses Enteroviruses Hepatitis A, E Polio	**C. Enteritis viruses** Hepatitis Poliomyelitis	8) *Clostridium difficile*	8) Bathwater	8) Diarrhea

Bacteriological Examination of Water

Bacteriological analysis of water supplies should be performed at regular intervals and not as a random exercise. The frequency of such analysis may range from daily to monthly sampling, depending on the size of the populations served.

Drinking water should be free of any pathogenic microorganisms. Ideally,

therefore, tests should be aimed at detecting such pathogens. However, these pathogens are generally present in such small numbers that they escape detection. The practice, therefore, is to test for **fecal pollution**; if fecal pollution is detected in a water sample, it is inferred that the water from which the sample was drawn may be harbouring enteric pathogens. An indicator of fecal pollution of water is the presence of **coliform bacteria** which are invariably present in large numbers in the feces of human beings and other warm-blooded animals and can be easily detected even in high dilutions. Detection of **thermotolerant *Escherichia coli*** provides definite proof of fecal pollution.

Other bacteria are also sometimes used as indicators of fecal pollution. These include 'fecal streptococci' (*Enterococcus*, resistant to 45°C, 40% bile, potassium tellurite and sodium azide concentrations inhibitory to coliforms) and *Clostridium perfringens.*

The following sections elaborate the tests that are generally done for the routine bacteriological analysis of water.

Plate count

Principle A count is made of the number of colonies formed in pour plate cultures of water samples, on nutrient agar incubated aerobically, in parallel, at 37°C for 1–2 days and at 22°C for 3 days.

Bacteria that grow at 37°C are most likely those associated with organic material of human or animal origin.

Bacteria that grow at 22°C are most likely to be saprobic and normally inhabit water or are derived from soil and vegetables.

Interpretation A plate count at 22°C provides an indication of the quantity of decomposing organic matter in the water; the greater the amount of organic matter present, the more likely the water is to be contaminated with parasitic and potentially pathogenic organisms. The plate count at 37°C is an important index of dangerous pollution—an increase in the plate count should serve as an indication of some defect in the filter beds, requiring immediate correction.

Detection of coliform bacteria and *Escherichia coli*

Presumptive coliform count—multiple tube technique

Concept This test is deemed presumptive because the reaction observed may occasionally be due to the presence of organisms other than coliform bacteria (hence the presumption that the reaction is due to coliform organisms has to be confirmed). An estimate of the number of coliform organisms is usually made by adding varying quantities of water (0.1–50 mL) to bile salt lactose peptone water (with an indicator for acidity) and incubating at appropriate temperatures.

Method The following range of quantities is usually prepared (Fig. 64.1):

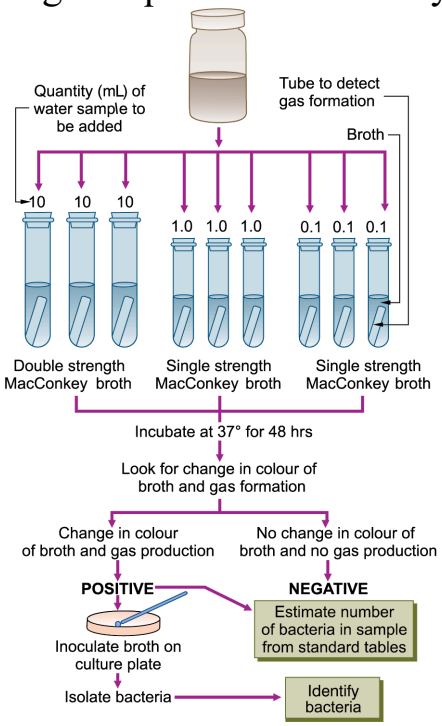

Quantity (mL) of water sample to be added
Tube to detect gas formation
Broth
10 10 10
1.0 1.0 1.0
0.1 0.1 0.1
Double strength MacConkey broth
Single strength MacConkey broth
Single strength MacConkey broth
Incubate at 37° for 48 hrs
Look for change in colour of broth and gas formation
Change in colour of broth and gas production
No change in colour of broth and no gas production
POSITIVE
NEGATIVE
Inoculate broth on culture plate
Estimate number of bacteria in sample from standard tables
Isolate bacteria
Identify bacteria

Fig. 64.1 Standard method for bacteriological analysis of water

- One 50 mL quantity of water added to 50 mL double-strength medium
- Five 10 mL quantities each to 10 mL double-strength medium
- Five 1 mL quantities each to 5 mL single-strength medium
- Five 0.1 mL quantities each to 5 mL single-strength medium

MacConkey's liquid medium (modified) is used.

Interpretation The probable number of coliforms per 100 mL is read from the probability tables of McCrady. This is known as the 'presumptive coliform count' or the most probable number (MPN) of coliforms present in

the water sample being tested (Table 64.2).

Table 64.2 Classification of the quality of drinking water based on bacteriological tests

Classes	Presumptive coliform count per 100 mL	*E. coli* count per 100 mL
Class I—Excellent	0	0
Class II—Satisfactory	1–3	0
Class III—Suspicious	4–10	0
Class IV—Unsatisfactory	More than 10	0.1 or more

Differential coliform test (Eijkman test)

Concept This test is usually employed to determine whether the coliform bacilli detected in the presumptive test are, in fact, *E. coli*.

Method Following the presumptive test, subcultures are made from all the bottles showing acid and gas to fresh tubes of **single-strength** MacConkey medium pre-warmed to 37°C. These tubes are **incubated at 44°C** (this is done in thermostatically controlled water baths that do not deviate more than 0.5°C from 44°C) and examined after 24 hours.

Interpretation Tubes exhibiting gas in Durham's tubes are deemed to contain *E. coli*. From the number of positive tubes obtained, the number of *E. coli* bacteria present in the water sample tested can be estimated by referring to McCrady's probability tables. The presence of *E. coli* can be further confirmed by testing for indole production and citrate utilisation.

Membrane filtration method

Concept A measured volume of water is filtered through a membrane filter with a pore size of 22 µm (bacteria-stopping filters). Bacteria, if present, are retained on the surface of the filter.

Detection of fecal streptococci

Principle Fecal streptococci (such as the *Enterococcus* species) may be present in water samples but usually survive only for short durations. Hence if fecal streptococci are detected in water after 45°C in a water sample, it suggests recent fecal contamination of the water.

Detection of *Clostridium perfringens*

Principle *C. perfringens* may be present in water samples over a long duration. Hence if *C. perfringens* alone is detected in a water sample (without *E. coli* or fecal streptococci), it suggests fecal contamination that is not recent.

Method Varying quantities of the water samples are incubated in litmus milk medium (anaerobically) at 37°C for five days; '**stormy clot**' formation is looked for, which indicates the presence of *C. perfringens*.

Tests for pathogenic bacteria

Under special circumstances, specific pathogenic bacteria, such as typhoid bacilli or cholera vibrios, may have to be looked for in water.

A simple and sensitive method is to filter the water sample through membrane filters and to then incubate the filters on appropriate solid media.

Viruses in Water

Enteroviruses and other cytopathogenic viruses can be isolated from water but this is not routinely done. Generally, it is assumed that viruses in water are destroyed by chlorination, wherein the concentration of free residual chlorine is at least 0.5 mg per litre for a minimum contact period of 30 minutes at pH below 8 and a turbidity of 1 nephelometric turbidity unit or less.

Protozoa in Water

Entamoeba histolytica, the *Giardia* species and *Balantidium coli* may contaminate drinking water. Coliform counts do not reliably indicate protozoal contamination of chlorinated water as protozoa are more resistant to chlorine than coliforms.

BACTERIOLOGY OF AIR

Definitions

Airborne infection Transmission of infection produced by respiratory droplets less than 5 μm in size

Droplet infection Transmission of infection produced by respiratory droplets larger than 5 μm in size

Microbial Content of Outdoor Air

This depends on many **factors,** including the density of human and animal populations, the nature of the soil, the density of vegetation and atmospheric conditions (humidity, temperature, wind conditions, rainfall and sunlight).

Bacteria in the upper air comprise largely of aerobic spore-bearing bacilli and, to a lesser extent, *Achromobacter*, *Sarcina* and *Micrococcus.* **Infective microbes are seldom carried for more than short distances** and their capacity to cause infection is impaired, except in rare cases such as the foot-and-mouth disease virus. **Pathogenic bacteria do not multiply in air.**

Microbial Content of Indoor Air

Here, bacteria may be distributed through gross droplets and droplet nuclei from the nose and mouth and through dust particles.

Dust consists of particles of varying sizes originating from animal, vegetable or mineral sources. The ultimate source of common pathogenic organisms is dust from human beings.

- Nasal secretions via the ala nasi and upper lip are carried by the hands to the skin, clothing and bedding from where they become detached as dust particles.
- Organisms may also get directly detached from the skin of different parts of the body including the perineum and septic wounds.
- Intestinal organisms, through dried particles of feces from napkins of infants, are also disseminated.

Heavy particles fall to the ground, while those 1 mm or less in diameter mostly remain suspended in the air. **Hemolytic streptococci** from patients or carriers, **tubercle bacilli** and **diphtheria bacilli** and **staphylococci** are found in the dust of wards where such patients are treated. Under favourable conditions, they may remain alive for many weeks. Bedclothes are an abundant source of bacteria-laden dust.

Droplets and droplet nuclei While **coughing, sneezing and talking**, varying numbers of droplets are expelled from the body, ranging in size

from less than 1 mm to
15 mm (Table 64.3).

Table 64.3 Pathogenic microorganisms that spread through air

Features	Droplet transmission	Airborne transmission
Size of droplet	> 5 μm in size (droplet nuclei)	< 5 μm in size
Source of droplets	Produced during coughing, sneezing, talking, invasive procedures (e.g., bronchoscopy)	Produced during coughing, talking, sneezing, invasive procedures (bronchoscopy, suction aspiration)
Characteristics of droplets	Droplet nuclei arise due to evaporation Present in air for short time and travel only short distances (≤1 m) Close contact needed for this mode of transmission	Remain suspended in air for long periods Travel several metres Susceptible individual may become infected even if there is some distance from infected person
Microorganisms involved a) Bacteria	*Streptococcus pyogenes* *Neisseria meningitidis* *Corynebacterium diphtheriae* *Haemophilus influenzae* type B *Bordetella pertussis* *Yersinia pestis* (pneumonic plague) *Mycoplasma pneumoniae*	*Mycobacterium tuberculosis*
b) Viruses	Influenza viruses Rubella virus Mumps virus Adenovirus Parvovirus B19	Varicella-zoster virus Measles virus Influenza viruses

Note:
1. *S. aureus* and *S. pyogenes* are known to be shed and dispersed into air in operating rooms and newborn nurseries.
2. Outbreaks of pneumonia due to *Legionella pneumophila* are associated with the presence of cooling towers close to the ventilation systems of hospitals.
3. *Aspergillus* and other fungal spores are dispersed through the air during construction, renovation and maintenance of buildings.
4. Although carpets, linen, potted plants and flowers are known to be reservoirs of opportunistic pathogens, epidemiological evidence linking these to nosocomial infections is lacking.

The air quality of operation theatres (OT) in hospitals has to be kept under control. It depends on the type of (OT): specialty OT, General OT and Minor (day-care) OT.

Factors that are regulated in an OT through air handling units (AHU) and high efficiency particulate air (HEPA) filters are the following:

- Velocity
- Air change/hour
- Positive pressure
- Air filtration
- Temperature and humidity

Measurement of Air Contamination

Sedimentation 'settle plate' method

Definition A means of estimating the number of bacteria present in the air is by permitting bacteria to 'settle' on open petri dishes (containing culture media) over a fixed duration. Droplet nuclei require more time to settle than larger particles.

Method Open plates of culture media are exposed for specific periods, for example, half to one hour; then the plates are incubated at 37°C for 24 hours and the number of colonies counted.

Interpretation This method provides an idea of the relative numbers and species of microorganisms present in air and is specially used for testing the quality of air in **surgical theatres and hospital wards.**

Slit sampler

Definition This is a method of estimating the number of bacteria present in the air by passing a known volume of air through a 'slit'.

This is a more elaborate method than the plate exposure method and makes up for the many limitations of the latter. In this, a known volume of air is directed onto a plate through a slit 0.25 mm wide, the plate being mechanically rotated so that the organisms are evenly distributed over it.

BACTERIOLOGY OF MILK

Types of Bacteria in Milk

i) ***Acid-forming bacteria*** The commonest are lactic streptococci including *S. lactis* and *Enterococcus faecalis*. Lactobacilli are also found. These ferment the lactose in milk, producing acids, mainly lactic acids, which lead to the formation of a smooth, gelatinous curd.

ii) ***Alkali-forming bacteria*** These consist of the *Alcaligenes* spp., some aerobic spore bearers and the *Achromobacter* species. These render the milk alkaline.

iii) ***Gas-forming bacteria*** Coliform bacilli are the commonest. Others are *C. perfringens* and *C. butyricum*. They produce acid and gas—a smooth, gelatinous curd riddled with gas bubbles is formed. Coliform bacilli are responsible for the ropiness in milk.

iv) ***Proteolytic bacteria*** Spore-bearing aerobes such as *Bacillus subtilis* and *Bacillus cereus*, *Proteus vulgaris,* staphylococci and micrococci come under this category.

v) ***Inert bacteria*** These are bacteria that produce no visible change in milk. These include some cocci of the udder, members of the *Achromobacter* group and most of the pathogenic organisms in milk.

vi) ***Human milk*** Breast milk contains small numbers of *S. epidermidis, S. mitis, Gaffkya tetragena* and *S. aureus*. A few other species may also be found in some samples.

Milk-Borne Diseases

Occasionally, milk may be contaminated with *Streptobacillus moniliformis* from the nasal secretions of rats and with *Campylobacter jejuni* from animal feces. *Yersinia enterocolitica* is not uncommon in milk and may give rise to gastroenteritis if present in large numbers (Table 64.4).

Table 64.4 Pathogenic microorganisms that spread through milk

Organisms derived from infected cow			Organisms from sources external to animal	
Organism	**Disease in cow**	**Disease in humans**	**Organism**	**Disease in humans**
A) Bacteria			A) Non-pathogenic organisms	
1. *Mycobacterium bovis*	1. Tuberculosis	1. Tuberculosis	1. Non-chromogenic lactic streptococci	1. Non-pathogenic but may cause unpleasant taste and odour of milk
2. *Bacillus anthracis*	2. Anthrax	2. Anthrax	*Lactobacillus* spp.	2. Non-pathogenic but may cause changes resulting in milk of blue, red or yellow colour
3. *Brucella abortus*	3. Brucellosis	3. Undulant (remittent) fever	*Bacillus subtilis*	
4. *Streptococcus* spp.*	4. Mastitis	4. Sore throat, scarlet fever	2. Chromogenic bacteria *Chromobacterium violaceum*	
5. *S. aureus*	5. Mastitis	5. Diarrhea, vomiting		
6. *Salmonella* spp.*	6. Bacteremia	6. Enteric fever, food poisoning		
7. *Coxiella burnetii***	7. Q fever	7. Q fever		
B) Viruses			B) Pathogenic bacteria **	
1. Cowpox virus	1. Vesicles on cow udder	1. Lesions on skin of milker	1. *Shigella* spp.	1. Dysentery
2. Foot-and-mouth disease virus	2. Foot-and-mouth disease	2. Mild disease (fever, vesicles on skin and mucous membranes)	2. *Vibrio cholerae*	2. Cholera
			3. *Corynebacterium diphtheriae*	3. Diphtheria
			4. *S. pyogenes*	4. Scarlet fever
			5. Diarrheagenic *E. coli*	5. Diarrhea
			6. Salmonella	6. Enteric fever
			7. *S. aureus*	7. Food-poisoning
			C) Pathogenic viruses**	
			1. Polioviruses	1. Paralytic poliomyelitis
			2. Hepatitis A virus	2. Infective hepatitis

* May be spread through milk in the form of ice cream

** Milk may not be the usual vehicle of spread of these pathogens

Sterilisation of Milk

Potential pathogens are eliminated from milk by the following methods:

1. ***Pasteurisation*** In this method, all vegetative pathogens are killed by heating milk to 63–66°C and maintaining it at this temperature for 30 minutes (the holder method) or by heating milk to a temperature of 71°C and holding it at that temperature for at least 15 seconds. The second step of this method is the rapid cooling of milk to 10°C or less. The phosphatase test is used to check the adequacy of pasteurisation.
2. ***Boiling*** The commonly-used household method of heating milk at or around boiling point (which destroys all but the most resistant spores) is adequate for short-term purposes. The efficacy of such treatment is tested by the turbidity test.
3. ***Ultraheat treatment*** In this method, milk is heated to 132°C for one second under specified conditions.

Bacteriological Examination of Milk

Routine bacteriological examination of milk consists of the following:

Viable count

Method This is estimated by performing plate counts with serial dilutions of the milk sample. Raw milk always contains bacteria, varying in number from about 500 to several million per mL.

Significance The plate count gives a rough and direct assessment of the viable bacteria in a sample of milk. It is easily explainable to the producer and gives a fair idea of the improvement or deterioration in the conditions of production.

Test for coliform bacilli

The coliform test is a useful indicator of fecal contamination and also of contamination by dust or unclean utensils.

Methylene blue reduction test

Method This is a simple substitute for the viable count. It depends on the reduction of methylene blue by bacteria in milk when incubated at 37°C in complete darkness.

Significance The rate of reduction is related to the degree of bacterial contamination. Raw milk is considered satisfactory if it fails to reduce the dye in 30 minutes.

The **resazurin test** is similar, except that the dye resazurin, on reduction, passes through a series of colour changes—from blue to pink to colourless. The colour change is noted after incubation with the milk for 10 minutes.

Phosphatase test

Method This is a check on whether milk has been pasteurised. The enzyme phosphatase, which is normally present in milk, is inactivated if pasteurisation has been performed properly.

Examination for specific pathogens

Tubercle bacillus The milk is centrifuged at 3,000 rpm for 30 minutes and the sediment inoculated into two guinea pigs.

Brucella Isolation of brucella may be attempted by inoculating cream

heavily on serum dextrose agar or by injecting a centrifuged deposit of the milk sample intramuscularly into guinea pigs. The animals are sacrificed after six weeks and the serum tested for agglutinins and the spleen inoculated in culture media. Brucellosis in animals can also be detected by demonstrating the antibodies in milk by the milk-ring or the whey agglutination tests.

The tests adopted for the routine examination of milk should reveal the degree of bacterial contamination and thereby indicate whether the milk is produced and handled in a hygienic manner.

BACTERIOLOGY OF FOOD

Food-borne infections are a significant public health problem since they may be a major cause of morbidity, although, fortunately, an infrequent cause of mortality. Food is very easily contaminated and, in addition, is an excellent medium for the growth of various kinds of microorganisms.

Etiologic patterns of food-borne infections vary across the world. In the USA, *S. aureus* and *Salmonella* are responsible for more than half the food-borne outbreaks of disease, whereas in the United Kingdom, *Clostridium perfringens* is responsible for more than 90 per cent and in Japan, *Vibrio parahaemolyticus* is responsible for more than 50 per cent of such outbreaks.

Definitions

Outbreak of food-borne disease This is said to have occurred when two or more persons experience a similar illness, usually gastrointestinal, after the ingestion of the same food and epidemiologic analysis implicates the food as the source of the illness.

Food poisoning A group of diseases caused by the consumption of food contaminated with microbes or microbial toxins (usually caused by bacteria).

Source of Food Contamination

Food should not normally contain microorganisms. If microbes are detected in food, particularly in cooked food, it indicates a breakdown of sanitary procedures and precautions. Food can be contaminated by the following means:

- ***Feces*** Food may get directly contaminated with feces through the hands of the infected person or indirectly through objects which he has handled.
- ***Sewage*** Water used for cooking may have been contaminated by sewage at some stage.
- ***Flies*** These insects carry pathogenic organisms from feces or from waste in refuse dumps or dustbins to food; they also contaminate food by their excreta.
- ***Hands of infected individuals*** Food may be contaminated by the hands of individuals who are either infected or are carriers and who handle food.
- ***Air*** Uncovered food may be contaminated by organisms present in air and dust which settle on it.
- ***Domestic animals and pets*** Food may inadvertently be contaminated by the excreta of domestic animals and pets, either directly or indirectly (through the hands of persons handling the animals).

Laboratory Diagnosis of Suspected Food-Borne Infection or Food Poisoning

Clinical history Since time is of the essence, a careful and detailed history should be taken, to eliminate unlikely causes altogether.

Samples for investigation Samples include the food consumed, and the vomitus and feces of the affected person.

Investigations on samples

- Direct microscopic examination for the presence of parasites
- Culture of the samples for bacteria and other microorganisms

Tests for detection of bacterial toxins Food products are analysed to determine the total microbial count and to detect the presence of coliform bacilli (similar to the principles followed in the microbiological analysis of water).

Prevention

Food may be contaminated prior to cooking. The contamination may be because the food has been taken from an infected animal or because the food may be contaminated at the time of the animal's slaughter in the abattoir or when it is canned improperly. To overcome this, care should be taken while cooking to ensure that heat **penetrates all parts** of the food so that all bacteria are killed.

Refrigeration does not kill microorganisms; it only serves to temporarily inhibit their growth.

Reheated foods are often sources of food poisoning, particularly if they have been contaminated by enterotoxin-producing *S. aureus*, since the heat does not affect the heat-stable enterotoxin secreted by the bacterium. Imperfectly or undercooked food, for example, meat that has been broiled, could be a potential source of danger.

Overall hygiene practices must be maintained and the water used must come from protected or certified water supplies.

Key points

WATER

- Drinking water must be visually acceptable, clear and colourless, and without a disagreeable taste or odour.
- Water is a potential source of microbial diseases and may contain potential pathogens (species of *Salmonella*, *Shigella* and *Vibrio*, polioviruses and hepatitis A virus), suggesting contamination with human excreta (less commonly, animal or bird droppings).
- It is monitored by bacteriological methods to ensure its safety:
    - Plate count (total microbial count) at 22°C, if increased, suggests a large quantity of decomposing organic matter in the sampled water and increased risk of parasitic and potential pathogens being present.
    - Plate count at 37°C, if increased, suggests defective water-filtering beds requiring immediate remedy.
    - Increased presumptive coliform count suggests possible fecal contamination of a water supply.
    - A positive differential coliform count (even one *E. coli* present per 100 mL of water) confirms fecal contamination of a water supply, and strengthens the possibility of pathogens being present.
    - The presence of fecal streptococci suggests recent contamination, whereas the presence of *C. perfringens* alone (without *E. coli* or fecal streptococci) suggests fecal contamination that is not recent.

- Passing a known volume of the test water through a cellulose acetate membrane filter (22 µm pore size), which is then cultured, permits simultaneous determination of total counts, counts of coliform bacilli and counts of specific fecal *E. coli.*

AIR

- The bacterial content of the air is important, particularly when pathogens are present. Heavy particles fall to the ground; particles <1 mm in size remain suspended in air.

MILK

- Human milk is seldom a vector of pathogens. Cow's milk may contain pathogens excreted in the milk or derived from the animal's udders (*Mycobacterium tuberculosis* and *Brucella abortus*) or from the animal's feces (salmonellae and campylobacters).
- Regular tuberculin testing of cattle and examination of milk for brucella antibodies enables the detection and slaughter of infected animals.
- To eliminate potential pathogens, milk is treated by:
    - Pasteurisation, where milk is heated to 63–66°C, kept at this temperature for 30 minutes (the holder process) or heated to 71°C (at least 15 seconds) to kill all vegetative pathogens, and then rapidly cooled to 10°C or less. The phosphatase test is used to check the adequacy of pasteurisation.
    - Heating at or around boiling point (destroys all but the most resistant spores) is adequate for less long-term purposes. The efficacy of such treatment is evaluated by the turbidity test.
    - Ultraheat treatment, where milk is heated to 132°C (1 second under specified conditions) is another method used.

FOOD

- Food-borne infections are serious causes of morbidity.
- Food may be contaminated by feces, sewage, flies, infected hands, air, and domestic animals and pets. Food poisoning may be caused by toxin-producing organisms (*Clostridium botulinum*) and invasive organisms (non-typhoid salmonellae).

Questions

Essay

1. Describe milk-borne pathogens. How will you eliminate them?

Short Answers

1. Air sampling
2. Coliform count

3. Water analysis to check waterborne infections in a hospital

Part VI: Clinical Microbiology as Applied to Infectious Diseases

Chapter 65

Biomedical Waste Management

Chapter navigation

- Types of biomedical waste
- General principles of waste management
- Waste treatment
- BMW 2016 Rules

INTRODUCTION

Hospitals generate large quantities of potentially hazardous waste daily. They may be infectious and non-infectious materials.

Definition **Biomedical or hospital waste** refers to any waste generated while providing health care, performing research and undertaking investigations or related procedures on human beings or animals in hospitals, clinics, laboratories or similar establishments (Management and Handling Rules: Government of India, 2016).

Potential hazards Biomedical waste (BMW) is far more dangerous and offensive than domestic waste because:

- It contains infectious or other hazardous materials that may injure, infect or otherwise harm patients, their visitors, hospital personnel and the public at large in several ways.
- It may contain ‘sharps’, such as needles or broken glass, that can cause injury and infection, or harmful chemicals and radioactive materials.
- If left untreated, biomedical waste undergoes fermentation and tends to attract flies and other insects, birds and animals,

rendering the site of generation and storage filthy and unhygienic.
- It can attract rag pickers, who repack disposables or drugs and sell them.

Since biomedical waste materials can be serious pollutants of soil, water and air unless carefully managed, governments across the world have been forced to introduce legal restraints in this area. The Government of India has promulgated the **Medical Waste (Management and Handling) Rules, 1998,** under which, persons who are in charge of medical and other institutions where such wastes are generated (called **'occupiers'**) are held legally responsible for maintaining the conditions prescribed in the rules. These rules came into effect on 1 January 2003.

The following practices are mandatory for all health care settings.

- Colour-coded bags for infectious, non-infectious and general waste
- Ban on the use of chlorinated plastic bags and gloves beyond 27 March 2019
- Puncture-proof containers for sharps and needles
- Pre-treatment, autoclaving of infectious waste (blood and body products) prior to disposal
- Deep burial and incineration of appropriate hospital waste in a central facility

TYPES OF BIOMEDICAL WASTE

The amount of biomedical waste generated under Indian conditions has been estimated to be 1 to 2 kg per bed per day and is composed of different types of waste, of which, on an average, about 85 per cent is harmless and only 15 per cent is hazardous, which includes infectious waste (Table 65.1).

Table 65.1 Types of waste in health care setting

Type of waste	Materials
Harmless waste	Paper, cardboard, cartons, flowers and ordinary office or kitchen waste (akin to domestic waste)

Infectious waste	• Human or animal tissues or organs removed during biopsy, surgery or autopsy • Placenta and other products of conception • Any pathological fluid or discharges • Dressings, swabs and other soiled items • Laboratory samples sent for microbiology, pathology and biochemical tests
Non-infectious hazardous waste • Chemical • Radioactive • Pharmacological	Toxic, corrosive, inflammable, reactive and otherwise injurious waste, the handling and management of which are under the direction of the Bhabha Atomic Research Centre
	Surplus or time-expired drugs

GENERAL PRINCIPLES OF WASTE MANAGEMENT

Effective waste management requires a clean and tidy environment since waste tends to accumulate in dirty surroundings. The hospital and its premises should be maintained in a clean and hygienic state. This requires frequent soap and water washing, mopping and good housekeeping practices. The objectives of biowaste management are to prevent harm resulting from waste, minimise its volume, retrieve reusable materials and ensure safe and economical disposal.

STEPS IN WASTE MANAGEMENT

1. **Reduction** in volume can be achieved by proper planning and using reusable items wherever safely possible.
2. **Segregation** refers to the separation of waste at the point of generation into the various types with respect to their category and mode. When potentially harmful waste is mixed with harmless waste such as paper or packing materials, the entire load becomes contaminated and will have to be autoclaved, instead of only the originally infected items. Segregated waste is to be put into different coloured containers, as prescribed in the rules, for appropriate treatment. These guidelines were modified in 2018. The colour coding is shown in Table 65.2.
3. **Storage** refers to the measures taken to ensure that biomedical waste is kept safely at the point of generation before being sent to the

biomedical waste treatment facility.

4. **Transportation** refers to the movement of waste from the point of generation to the treatment facility.
5. **Treatment** here means all the procedures and processes intended to reduce the bulk of the waste and make it non-infectious and harmless.

METHODS OF WASTE MANAGEMENT

Several methods of waste treatment are available and the choice of method depends on the item of waste and the facilities available. The place of final disposal may be on the premises or, if possible, away from crowded areas. Some methods of waste treatment are indicated below.

Chemical disinfection This is a very useful method for many items, particularly in small health care settings such as clinics. For some materials, it is also an important preliminary process before final treatment. For example, contaminated materials, such as sputum or pus, must be disinfected before being buried or autoclaved.

Deep burial Where large areas of uninhabited land are available, this is a convenient method for waste treatment. After chemical disinfection, materials are placed in deep trenches, covered with lime and the trenches filled with soil. This is also a safe method for the disposal of sharps.

Incineration This is a safe method to treat large solid infectious waste, particularly anatomical waste and amputated limbs, animal carcasses and similar materials. The incinerator exposes the waste material to a very high temperature, converting the material into ash, which would be only about a tenth of the original volume. However, it is expensive and is generally used only by very large establishments.

Autoclaving This is widely used in laboratories and clinics for treating infectious waste before disposal.

Microwaving This is another useful method of sterilisation of small-volume waste at the point of generation. This cannot be used for animal or human body parts, metal items or toxic or radioactive material.

Inertisation This process involves mixing waste with cement and other substances before disposal, in order to minimise the risk of toxic substances

contained in the wastes migrating into the surface water or groundwater. This method is usually adopted for pharmaceutical waste, pills and drugs.

Encapsulation In this method, plastic or steel drums are filled to 75% capacity with solid and semi-solid pharmaceuticals and the remaining space is filled with cement or a cement–lime mixture. The drum is then sealed and buried in the bottom of a land fill.

Liquid waste Pathological, chemical and toxic liquid waste should be appropriately treated with disinfectants or reagents and neutralised before flushing them into the sewer.

Proper disposal of hospital waste constitutes an important measure to prevent health care-associated infections. After all, the first rule in medicine as well as nursing is ***Primum non nocere,*** meaning, **"First, do no harm".**

SPILL MANAGEMENT

Exposure to blood and other body fluids poses a risk of infection. Management of spillages is an essential part of prevention and control of infections like HIV, hepatitis B and hepatitis C through blood or other body fluids. These fluids can be hazardous to the health care persons, besides being a source of infection to other patients. Management of blood and body fluid spillage is one of the standard infection control practices. It must be assumed that every individual could be carrying potentially harmful microorganisms.

Procedure for Spill Management

The spillage material includes blood and/or other body fluids, CSF and peritoneal fluids which are potential sources of infection.

A spill kit is handy for managing a spill. A paper-towel is placed on the spillage and 1% sodium **hypochlorite solution is added** to the spillage so that it completely covers the spillage (10,000 ppm). It is left on for 2 to 5 minutes for adequate contact time. The fluid is disposed along with the paper towels into the yellow bin. The area is cleaned with detergent and water using disposable cloth and dried thoroughly. The staff must wear PPE (personal protective equipment) while cleaning (gloves, mask and gown) and perform decontamination of their hands afterwards.

BMW RULES 2016, AMENDED IN 2018

These were published in the Gazette of India, and amended in 2018 to include:

i) Phasing out of chlorinated plastic bags (excluding blood bags) and gloves by 27 March 2019

ii) Disposal in accordance with the guidelines issued by the Central Pollution Control Board

(The rules for biomedical waste management were further amended in 2019 to include monthly and annual reports on the websites of all hospitals with in-patients.)

The major change in segregation is the change in the colour code and the waste categories (Table 65.2).

Table 65.2 Colour-coded bags for biomedical waste segregation

Colour of the bag	Type of waste	Waste treatment
Yellow	a) Human anatomical waste	Incineration or plasma pyrolysis or deep burial
	b) Animal anatomical waste	
	c) Soiled waste	
	d) Expired or discarded medicines	Returned to the manufacturer or supplier for incineration at temperature >1,200°C
	e) Chemical waste	Incineration, plasma pyrolysis, deep burial or encapsulation
	f) Chemical liquid waste	Pre-treatment and then disposal
	g) Discarded linen, mattresses, beddings contaminated with blood or body fluids	Non-chlorinated chemical disinfection followed by incineration or plasma pyrolysis
	h) Microbiology, biotechnology and other clinical laboratory waste	Pre-treat to sterilise with non-chlorinated chemicals on-site as per NACO or WHO guidelines and thereafter send for incineration
Red	i) Contaminated waste (recyclable) like plastic bag, bottles, pipes or containers	Autoclaving or microwaving/hydroclaving followed by shredding or mutilation Treated waste to be sent to registered or autoclaved recyclers or for energy

		recovery of plastics to diesel or fuel oil or for road-making
White, translucent	Waste sharps including metals: Needles, syringes with fixed needles, needles from needle tip cutter or burner, scalpels, blades	Autoclaving or dry-heat sterilisation; followed by shredding or mutilation or encapsulation in metal container or cement concrete Sent for final disposal to iron foundries (having consent to operate from the state pollution control committees) Or sanitary landfill or designated concrete waste sharp pit
Blue cardboard box with blue label or blue leak- and puncture-proof container	Glassware: Broken or discarded and contaminated glass including medicine vials and ampoules except those contaminated with cytotoxic wastes; metallic body implants	Disinfection (by soaking the washed glass waste after cleaning with detergent and sodium hypochlorite treatment) or through autoclaving or microwaving or hydroclaving, then sent for recycling

It is important to create awareness among health care workers and patients in the hospital regarding biomedical waste segregation at source and proper disposal. This can be done through posters (Fig. 65.1a and b) and training of the staff by the hospital infection control team.

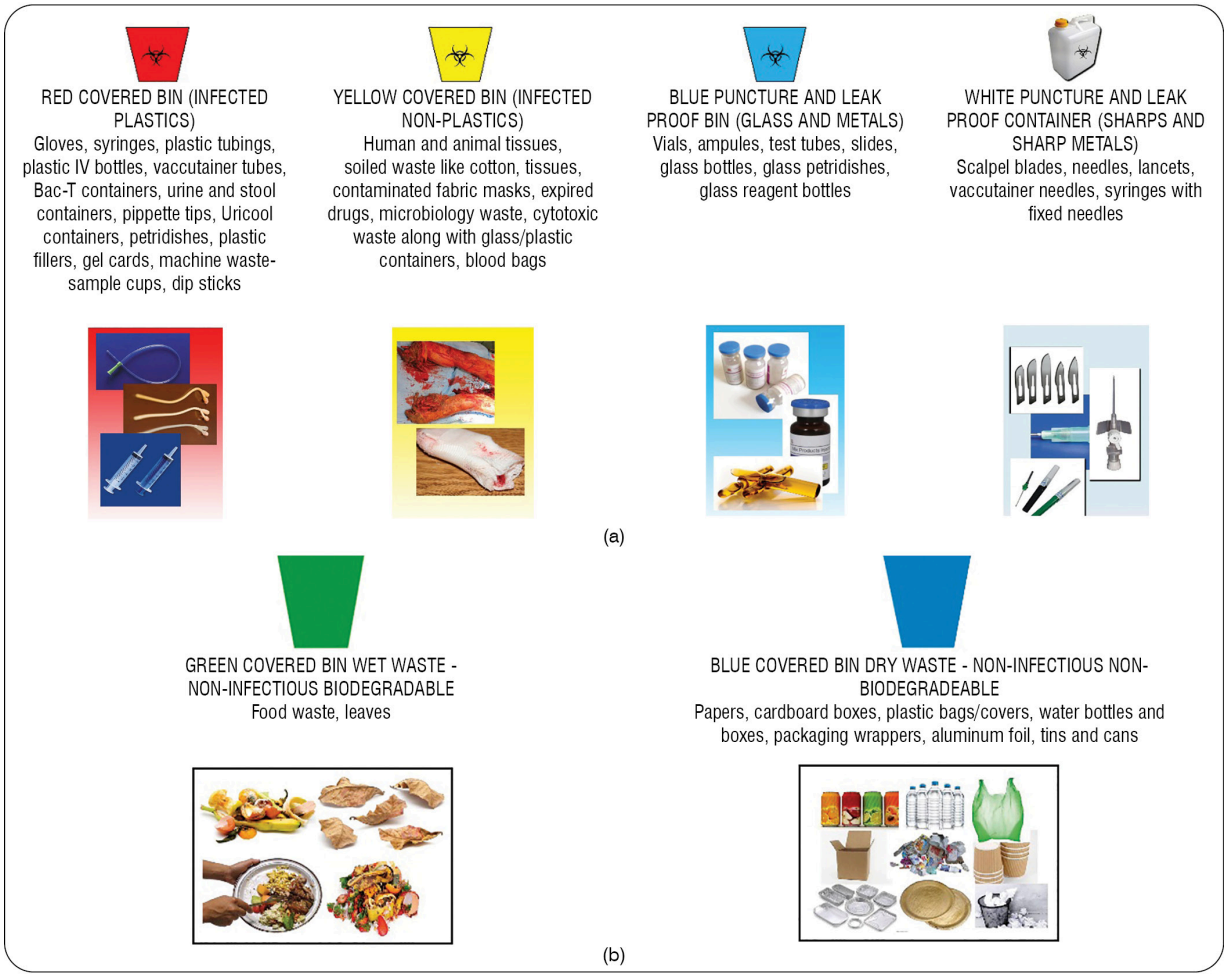

RED COVERED BIN (INFECTED PLASTICS)
Gloves, syringes, plastic tubings, plastic IV bottles, vaccutainer tubes, Bac-T containers, urine and stool containers, pippette tips, Uricool containers, petridishes, plastic fillers, gel cards, machine waste-sample cups, dip sticks
YELLOW COVERED BIN (INFECTED NON-PLASTICS)
Human and animal tissues, soiled waste like cotton, tissues, contaminated fabric masks, expired drugs, microbiology waste, cytotoxic waste along with glass/plastic containers, blood bags
BLUE PUNCTURE AND LEAK PROOF BIN (GLASS AND METALS)
Vials, ampules, test tubes, slides, glass bottles, glass petridishes, glass reagent bottles
WHITE PUNCTURE AND LEAK PROOF CONTAINER (SHARPS AND SHARP METALS)
Scalpel blades, needles, lancets, vaccutainer needles, syringes with fixed needles
(a)
GREEN COVERED BIN WET WASTE - NON-INFECTIOUS BIODEGRADABLE
Food waste, leaves
BLUE COVERED BIN DRY WASTE - NON-INFECTIOUS NON-BIODEGRADEABLE
Papers, cardboard boxes, plastic bags/covers, water bottles and boxes, packaging wrappers, aluminum foil, tins and cans
(b)

Fig. 65.1 Biomedical waste disposal posters (guidelines) displayed in hospitals to create awareness among health care workers: (a) infectious and (b) non-infectious waste (*Source:* Pondicherry Institute of Medical Sciences Hospital, Puducherry)

Key points

- Biomedical or hospital waste refers to any waste generated during health care, research, testing or related procedures on human beings or animals, conducted in hospitals, clinics, laboratories or similar establishments. Biomedical waste can be serious pollutants of soil, water and air. The Government of India has brought in legal restraints in this area by enacting the Medical Waste Management and Handling Rules, 2016.
- In Indian conditions, about 1–2 kg of waste per bed per day is generated, 15 per cent of which is hazardous. The different steps in waste management are reduction, reuse, segregation, storage, transportation and treatment. The methods of waste treatment, depending on the item of waste and the facilities available, include chemical disinfection, deep burial, incineration, autoclaving and microwaving.

- The most essential part of hospital waste management is the segregation of biomedical waste, which should be performed within the premises of the institution where the waste is generated.
- BMW 2016 rules have changed the colour codes and categories of waste.

Questions

Short Answers

1. Types of biomedical waste
2. Segregation of biomedical waste

Part VI: Clinical Microbiology as Applied to Infectious Diseases

CHAPTER 66

Quality Control and Recent Advances in Diagnostic Microbiology

Chapter navigation

- Automated and semi-automated systems
- Molecular methods in diagnosis
- Quality control (accreditation) and laboratory safety

INTRODUCTION

Conventional laboratory techniques for the diagnosis of infectious diseases, like culture and related methods, are widely used because they are sensitive and inexpensive. However, they are labour- and resource-intensive, requiring considerable expertise. They often require further characterisation by molecular techniques to confirm identification.

Molecular methods are useful in situations where conventional methods are slow, insensitive or cumbersome. Nucleic acid amplification tests and point-of-care molecular diagnostics, multiplexed panels for syndromic diagnosis, next-generation sequencing and automation of molecular tests, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) and mass spectrometry methods are some of the relatively recent advances in diagnostic laboratories.

These advances have decreased the turnaround time (from specimen collection to the final report reaching the patient) in culture, identification and antibiotic susceptibility testing.

AUTOMATED AND SEMI-AUTOMATED SYSTEMS FOR CULTURE

They fall into two main groups:

i) Automated culture systems, e.g., BacT/Alert and BACTEC systems

ii) Identification and susceptibility testing systems, e.g., VITEK and Phoenix systems

While some identification and susceptibility testing instruments take as long as traditional methods, others provide results within a single working day.

One of the newer methods to enter the chain is the **MALDI-TOF MS** (matrix-assisted laser desorption/ionisation–time of flight mass spectrometry). It is a technology used for the identification of any bacteria or fungi based on the unique protein composition of the bacterial cell. Its advantages are its rapidity (in minutes) and reliability. The drawback of the system at present is that it requires the organism to be cultured before identifying it.

The most recent advancement in diagnostic microbiology is the application of molecular techniques.

MOLECULAR METHODS

They can be broadly classified into three categories:

- Hybridisation
- Amplification
- Sequencing and enzymatic digestion of nucleic acids

Hybridisation

This is based on the ability of two nucleic acid strands that have complementary base sequences to bind specifically with each other and form a double-stranded molecule or hybrid. The assay requires one nucleic acid strand **(probe)** from an organism of known identity and another **(target)** from an unknown organism.

Hybridisation is detected by the use of a **reporter molecule** that forms a complex with the single-stranded probe DNA. Probes may be labelled with

a variety of molecules—radioactive, biotin-avidin and chemiluminescent labels. One of the major advancements of this technique has been in the application of line probe assay for the diagnosis of tuberculosis and detection of drug resistance.

Amplification

Polymerase chain reaction (PCR)

This is the most widely used target nucleic acid amplification method. It combines the principles of complementary nucleic acid hybridisation and replication.

Conventional PCR involves the following steps:

1. ***Extraction and denaturation*** The nucleic acid is first extracted from the organism or clinical sample by heat, chemical or enzymatic method, and then, the target nucleic acid is added to it and placed in a thermal cycler to undergo amplification.
2. ***Annealing*** Short strands of DNA sequences (primers) are selected to specifically hybridise (anneal) to a particular nucleic acid target.
3. ***Extension of the primer*** *Taq* polymerase is the enzyme commonly used for primer extension at a temperature of 72°C. Annealing provides a template that allows DNA polymerase to add nucleotides and produce, by extension, a sequence that is complementary to the target template.
4. ***Detection of PCR products*** The amplification product of PCR (amplicon) contains the target nucleic acid of interest. A labelled probe is used for detecting specific amplicons.

Types of PCR used in diagnostic microbiology

Multiplex PCR Two or more unique target sequences can be amplified simultaneously in this method. One primer pair is directed at sequences present in all clinically relevant bacteria (**control primer**) and the second is directed at a specific sequence (**test primer**). This is used to detect infectious agents in cases where the exact cause of the disease is not known.

Nested PCR This involves the sequential use of two primer sets. The first set is used for the amplification of a target sequence. The second set of primers is used to amplify a particular region within the first target

sequence. This gives a more specific test result and is used for specific disease detection. GeneXpert is widely used for detecting *M. tuberculosis* and resistance to rifampin and has become a mandated test by the Revised National Tuberculosis Programme (RNTCP) as a diagnostic tool and for the monitoring of drug resistance.

Quantitative PCR In addition to the detection of the causative agent of a disease, the actual number of targets in the clinical sample can be detected by this method, for example, detecting viral load in HIV.

Reverse transcriptase PCR (RT-PCR) Conventional PCR amplifies dsDNA but not RNA. The enzyme reverse transcriptase directs the synthesis of DNA from the viral RNA template. The DNA thus obtained is subjected to routine PCR. This is used to detect the H1N1 virus from nasopharyngeal swabs.

Real-time PCR This technique combines nucleic acid amplification with qualitative or quantitative measurement of the amplified product. Most molecular detection methods used in clinical microbiology use this technique.

Transcription-mediated amplification (TMA)

This is an isothermal RNA amplification method and uses three enzymes: RNase, DNA-dependent RNA polymerase and reverse transcriptase enzymes. The RNA target is converted to cDNA using reverse transcriptase and then RNA copies are synthesised with the help of RNA polymerase.

Nucleic acid sequence-based amplification (NASBA)

This is similar to TMA and is also an isothermal RNA amplification method. The RNA target is converted to cDNA using reverse transcriptase and then RNA copies are synthesised with the help of RNA polymerase.

Ligase chain reaction (LCR)

This reaction is based on the ligation of two adjacent synthetic oligonucleotide primers, which uniquely hybridise to one of the strands of the target DNA. A second pair of oligonucleotides is designed to hybridise to the complementary DNA, in the same region. When the nucleotides are present, the DNA polymerase and the ligase create a gap between the adjacent primers, which will then be filled with the appropriate nucleotides,

leading to the ligation of the primers. LCR allows the discrimination of DNA sequences differing in only a single base pair. Hence it is used to differentiate products.

Sequencing and Enzymatic Digestion of Nucleic Acid

Nucleic acid sequencing These are methods that determine the exact nucleotide sequence of a gene or gene fragment of an organism.

High-density DNA probes This uses hybridisation of a fluorescent-labelled nucleic acid target to a set of oligonucleotides synthesised on a miniature glass substrate or 'chip'.

Methods of typing isolates

Ribotyping This involves the enzymatic digestion of chromosomal DNA followed by hybridisation using probes that encode ribosomal RNA.

Restriction fragment length polymorphism (RFLP) Restriction enzyme analysis is a process by which enzyme digestion patterns are analysed. Restriction patterns are obtained after gel electrophoresis. The difference in restriction patterns of microorganisms is known as restriction fragment length polymorphism.

Pulsed-field gel electrophoresis (PFGE) Specialised electrophoresis devices are used to separate chromosomal fragments obtained by enzymatic digestion of intact bacterial chromosomal DNA. The DNA is then digested using restriction endonuclease enzymes.

The use of a pulsed electrical field across the agarose gel subjects the DNA fragment to different voltages from varying angles at different time intervals.

Applications of molecular methods

There is increasing application of molecular techniques in diagnostic microbiology. These methods have the following applications:

Diagnosis

- Detection of infectious agents from clinical specimens, CSF and body fluids
- Identification of bacteria and antimicrobial resistance (tuberculosis)
- Diagnosis of infections due to non-cultivable organisms

Epidemiology and disease detection

- Typing of isolates for epidemiological purposes and tracing their path of transmission

Research
- To understand the genetic make-up of infectious agents
- Drug discovery and newer diagnostics

QUALITY CONTROL AND ACCREDITATION OF LABORATORIES

Evidence-based diagnosis of diseases and management of patients depend on laboratory-confirmed diagnostic tests. Hence it is of utmost importance to practice and maintain strict quality-control and monitoring of processes involved in the functioning of the laboratories. Quality control of tests is maintained by ensuring accurate, reproducible and validated results of tests carried out in the laboratory.

All laboratory tests follow a common path of **pre-analytical** (pre-test), **analytica**l (test) and **post-analytical** (post-test) phases.

- Pre-analytical phase refers to the processes involved in the requisition of an investigation, collection of samples and transport to the laboratory where it is to be processed. There are standard operating procedures (SOPs), whereby each step adheres to a prescribed protocol.
- The analytical phase includes the actual processing of the sample which is crucial to the identification of the disease process. The standard procedures of testing, comparing with controls to validate the tests and correct interpretation of the results are part of this phase.
- Post-analytical phase refers to the reporting of results with correct transcription from the laboratory to the receiving of the results by the requisitioning physician.

These processes also include (i) adhering to laboratory safety and following safe practices while processing samples, (ii) use of biosafety cabinets to process infectious materials, (iii) calibration and safety of equipment to ensure accurate readings, (iv) training of laboratory personnel and (v) proper disposal of infectious and non-infectious materials.

The **National Accreditation Board for Testing and Calibration Laboratories (NABL)** is a constituent board of the Quality Council of

India. It adheres to the general requirements for the competence of testing and calibration laboratories, the ISO 15189. This document is the international standard, which is followed by all international accrediting bodies. The laboratories follow standard operating procedures for performing the tests and adhere to a set of quality guidelines which is specific for that laboratory.

Testing laboratories are assessed by the experts from NABL and if found to be compliant, the tests they perform are accredited. The assessment is done periodically to monitor the maintenance of strict quality procedures and the accreditation is renewed. The Govt. of India is in the process of making NABL accreditation mandatory for empanelling testing laboratories.

Key points

- Advances in microbial detection have been made by automated culture and susceptibility systems.
- Molecular methods are useful in situations where conventional methods are slow, insensitive or unavailable.
- The molecular methods used are hybridisation, amplification and sequencing, and enzymatic digestion of nucleic acids.
- In diagnostic microbiology, PCR, TMA, NASBA and LCR are commonly used for the detection of bacteria, fungi and toxins.
- Quality control in laboratories is necessary to provide reliable results.
- All laboratory tests follow a common path of:
    - pre-analytical (pre-test),
    - analytical (test) and
    - post-analytical (post-test).
- Laboratories are required to adhere to a set of guidelines based on the international quality standards and standard operating procedures. Testing laboratories are assessed by the experts from NABL and if found to be compliant, the tests they perform are accredited.

Questions

Essays

1. Explain the principle and steps of PCR.
2. Enumerate the different types of PCR used in clinical microbiology.
3. Describe the three phases of workflow in a diagnostic microbiology laboratory.

Short Answers

1. Principle of TMA
2. Principle of LCR

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